Trans-Endothelial Transport of High Density Lipoproteins in Inflammation and 3D Engineered Artery to Study Atherosclerosis

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Summary

Atherosclerosis is a chronic disease characterized by lipid retention and inflammation in the arterial intima. High density lipoproteins (HDL) and its major apolipoprotein (apoA-I) exert diverse potentially atheroprotective functions: For example, they reduce oxidative damage, correct endothelial dysfunction, inhibit inflammation and mediate reverse cholesterol transport. The latter process involves the removal of excess cholesterol from macrophage foam cells in the arterial wall and its delivery to the liver for biliary excretion.

This and others anti-atherogenic functions of HDL must be exerted in the arterial wall rather than in the plasma compartment. Therefore HDL or apoA-I must pass the endothelial barrier to get access to the foam cells. In previous work, it has been shown in cell culture experiments that endothelial cells transcytose HDL and apoA-I by distinct specific mechanisms involving the scavenger receptor (SR)-BI, the ATP binding cassette transporters (ABC) G1 for HDL and the ABCA1 as well as the ectopic β-ATPase for apoA-I. However little is known on how inflammation influences this trans-endothelium transport. Therefore we analyzed the role of the cytokines: interleukins (IL)-6, IL-1β and tumor necrosis factor (TNFα) on the trans-endothelial transport of HDL and apoA-I.

In the present thesis we demonstrated that the binding, internalization and trans-endothelial transport of HDL is influenced cytokine dependently. IL-6 increased the binding and transport of HDL without changing the expression of SR-BI and ABCG1. Therefore we characterized endothelial lipase (EL) a known HDL binding protein expressed by endothelial cells. Here we showed that IL-6 induces EL and that pharmacological inhibition of EL after IL-6 stimulation reverses the inducing effect of IL-6 on HDL binding and transport.

In addition upon stimulation of TNFα and IL-1β primary endothelial cells demonstrated a decrease of apoA-I binding and transport possibly due to decreasing cell surface expression of β-ATPase. Furthermore TNFα increased binding of HDL but reduced its transport. This discrepancy between binding and transport was not explained by enhanced cellular degradation but by reduced internalization.

The physiological relevance of in vitro studies is often contested. Therefore we characterized a novel 3D model based on the co-culture of endothelial cells and smooth muscle cells grown under flow condition. Histological analyses revealed that the artery equivalent mimick the structrure of a native artery. This raises the question, whether tissue engineered vascular grafts can serve as a model to study the development of atherosclerosis under flow conditions in vitro.

We demonstrated here the feasibility of this vascular graft model to record the insudation and accumulation of both low (LDL) and high density lipoprotein (HDL) within the arterial wall. In addition monocytes circulating through the system adhered to the endothelium after prelimary treatment with either TNFα or LDL and they transmigrated into the intima.

In conclusion our work suggests that inflammatory cytokines modulate HDL and apoA-I tansport through endothelial cells. In addition we revealed EL as a novel HDL binding partner in endothelial cells, which is modulated by cytokines and contributes to the trans-endothelial transport of HDL. Furthermore the novel engineered model appears to be suitable for studying atherosclerosis development in vitro.

Zusammenfassung

Atherosklerose stellt eine chronische Erkrankung dar, welche sich durch die Ansammlung von Lipiden sowie einer Entzündung im Bereich der inneren Gefässschicht von Arterien auszeichnet. High density lipoproteins (HDL) und sein wichtiges Apolipoprotein apoA-I haben diverse, potentiell atheroprotektive Funktionen: Zum Beispiel reduzieren sie oxidativen Stress, korrigieren endotheliale Fehlfunktion, hemmen Entzündung und könnten für den reversiblen Cholesterol-transport verantwortlich sein. Letzerer involviert die Entfernung von überschüssigem Cholesterol aus Makrophagen (sog. Schaumzellen) in den Arterienwänden und deren Transport zur Leber für die biliäre Ausscheidung. Diese und weitere anti-atherogene Funktionen von HDL werden in den Arterienwänden und nicht im Blutplasma vermutet. Daher müssen HDL und apoA-I die endotheliale Barriere passieren, um Zugang zu den Schaumzellen zu erlangen. Vorstudien konnten in Zellkulturexperimenten zeigen, dass Endothelzellen apoA-I und HDL via Transzytose mit Hilfe des Scanvanger Rezeptors (SR)-BI, des ATP binding cassette transporters (ABC) G1 für HDL, des ABCA1 sowie der ektopeṇ□-ATPase für apoA-I transportieren. Es ist jedoch wenig darüber bekannt inwiefern die Entzünung diesen transendothelialien Transport beinflussen kann. Deshalb untersuchten wir in unseren Experimenten die Rolle der folgenden Zytokine und deren Einfluss auf den transendothelialen Transport von HDL und apoA-I: (IL)-6, IL-1β, und tumor necrosis factor (TNFα).

Die vorliegende Arbeit zeigt, dass die Bindung, Internalisierung und der transendotheliale Transport von HDL von Zytokinen beeinflusst wird. Dabei erhöht IL-6 die Bindung und den Transport von HDL ohne jedoch die Expression von SR-BI und ABCG1 zu verändern. In der Folge charakterisierten wir die endotheliale Lipase (EL), welche ein bekanntes HDL-Bindungsprotein darstellt und durch Endothelzellen exprimiert wird. Wir konnten ferner zeigen, dass IL-6 EL induziert und die pharmakologische Hemmung von EL nach Stimulation mit IL-6 den induzierenden Effekt von IL-6 auf die HDL-Bindung bzw. den Transport umkehrt.

Zudem zeigten primäre Endothelzellen nach einer Stimulation durch TNFα und IL-1β eine Abnahme der Bindung und des Transportes von apoA-I, welche möglicherweise auf die Abnahme der Expression von β-ATPase zurückzuführen ist. Weiters beobachteten wir, dass TNFα eine Zunahme der Bindung von HDL bewirkte, jedoch dessen Transport verringerte. Diese Diskrepanz zwischen Bindung und Transport kann nicht mit erhöhtem zellulärem Abbau sondern vielmehr mit reduzierter Internalisierung erklärt werden.

Die physiologische Relevanz dieser in vitro Studien im Bereich der Atherosklerose-Forschung ist Gegenstand ausgedehnter Diskussionen und wird oftmals kritisiert. Wir haben deshalb ein neuartiges 3D-Modell entwickelt, welches auf der Co-Kultur von Endothelzellen und glatten Muskelzellen basiert und die Komponente des Flusses

miteinschliesst. Histologische Analysen dieser Konstrukte zeigten, dass die hergestellten Strukturen im Aufbau einer nativen Arterie ähneln. Dies entwirft die Fragestellung, ob diese „tissue engineerten“ Gefässe als Modelle für die Atheroskleroseforschung unter Einfluss von Flussbedingungen dienen könnten.

In der vorliegenden Arbeit konnten wir erste Schritte zur Entwicklung eines derartigen in vitro Modelles tätigen und zahlreiche Prozesse beobachten, welche im Rahmen der Atherosklerose-Entstehung in vivo relevant sind: Die Insudation von LDL und HDL in die Arterienwand, sowie die durch TNFα/LDL stimulierte Adhäsion und Transmigration von Monozyten.

Zusammenfassend zeigen diese Ergebnisse, dass pro-inflammatorische Zytkoine den Transport von HDL und apoA-I über die Endothelzellen modulieren. Zudem konnte gezeigt werden, dass EL einen HDL Bindungspartner in Endothelzellen darstellt und zum transendothelialen Transport von HDL beiträgt. Ausserdem zeigen die Ergebnisse, dass das neuartige 3D Modell für die in vitro Untersuchung von Atherosklerose zukünftig eine vielversprechende Option darstellen könnte.

List of abreviation

α-SMA:	α-smooth muscle actin
β-ATPase:	β-chain of F_0F_1 ATPase
ABCA1:	ATP binding cassett A1
ABCG1:	ATP binding cassett G1
Apo:	Apolipoprotein
CAD:	Cardiovascular disease
CD31:	Cluster of differentiation 31
CETP:	Cholesterol ester transfer protein
EC:	Endothelial cell
EL:	Endothelial lipase
eNOS:	Endothelial nitric oxide synthase
ER:	Endoplasmic reticulum
Col IV:	Type IV collagen
GAG:	Glycosaminglycan
HDL:	High density lipoprotein
HL:	Hepatic lipase
HSPG:	Heparan sulfate proteoglycan
HUVEC:	Human umbilical vein endothelial cells
Hyp:	Hydroxiproline
ICAM-I:	Intercellular adhesion molecule-I
IDL:	Intermediate low density lipoprotein
IL-1β:	Interleukin-1β
IL-6:	Interleukin-6
LCAT:	Lecithin/cholesterol acyltransferase
LDL:	Low density lipoprotein
LDLR:	LDL receptor
LLR:	LDL like receptor
LPL:	Lipoprotein lipase
LXR:	Liver X receptor
MCP-1	Monocyte chemotactic protein-1

MTP:	Microsomal triglyceride transfer protein
oxLDL:	oxidized LDL
PLTP:	Phospholipids transfer protein
SR:	Scavenger receptor
SR-BI:	Scavenger receptor-BI
SREBP	Sterol regulatory element-binding protein
TNFα:	Tumour necrosis factor α
UCMFB:	Human umbelical myofibroblast
VCAM-I:	Vascular cell adhesion-I
VLDL:	Very low density lipoprotein
ZO-1:	Tight junction protein 1

Chapter

1

General Introduction

Vascular physiology

The cardiovascular system is responsible for the transport of nutriments, gazes and blood cells through the entire body. While the heart represents the biomechanical pump creating the force to trigger blood flow, blood vessels transport the blood throughout the entire body. There are three major types of blood vessels; the arteries, transporting the blood away from the heart, the capillaries, enabling the exchanges between blood and cells and finally the veins, carrying the blood back to the heart.

The arteries are of particular interest because they represent the predispose sites of atherosclerosis development [1]. Arteries are built up of three layers (or tunica); i) the tunica intima composed of endothelial cells lining the vessel lumen, ii) the tunica media essentially composed of smooth muscle cells and iii) the tunica adventitia consisting of connective tissue and anchoring the artery in the surrounding tissue (Fig. 1).

i) ***The tunica intima*** (or intima) is the innermost layer of the artery and limits the vessel wall towards the lumen. It is composed of a monolayer of endothelial cells demonstrating anti-thrombotic properties as well as semi-permeable barrier function by the formation of tight and adherens junctions between the cells. Endothelial cells produce in their basolateral side a thin continuous extracellular matrix, the basement membrane. This special extracellular matrix is essentially composed of type IV collagen, laminin and perlecans [2]. In addition a layer of elastin, the internal elastic membrane, is separating the intima to the media.

ii) ***The tunica media*** (or media) is the middle layer and consists primarily of one or multiple layers of circumferential arranged smooth muscle cells. Variable amounts of elastin, proteoglycans and collagens are located between the smooth muscle cells. The external elastic membrane separates the media to the adventitia.

iii) ***The tunica adventitia*** (or adventitia) forms the outmost connective tissue layer of the vessel. It is composed of fibroblasts and longitudinally arranged

collagenous tissues anchoring the vessel to the surrounding tissues. In addition the adventitia contains small blood vessels (vasa vasorum) supplying blood to the vascular wall as well as nerves.

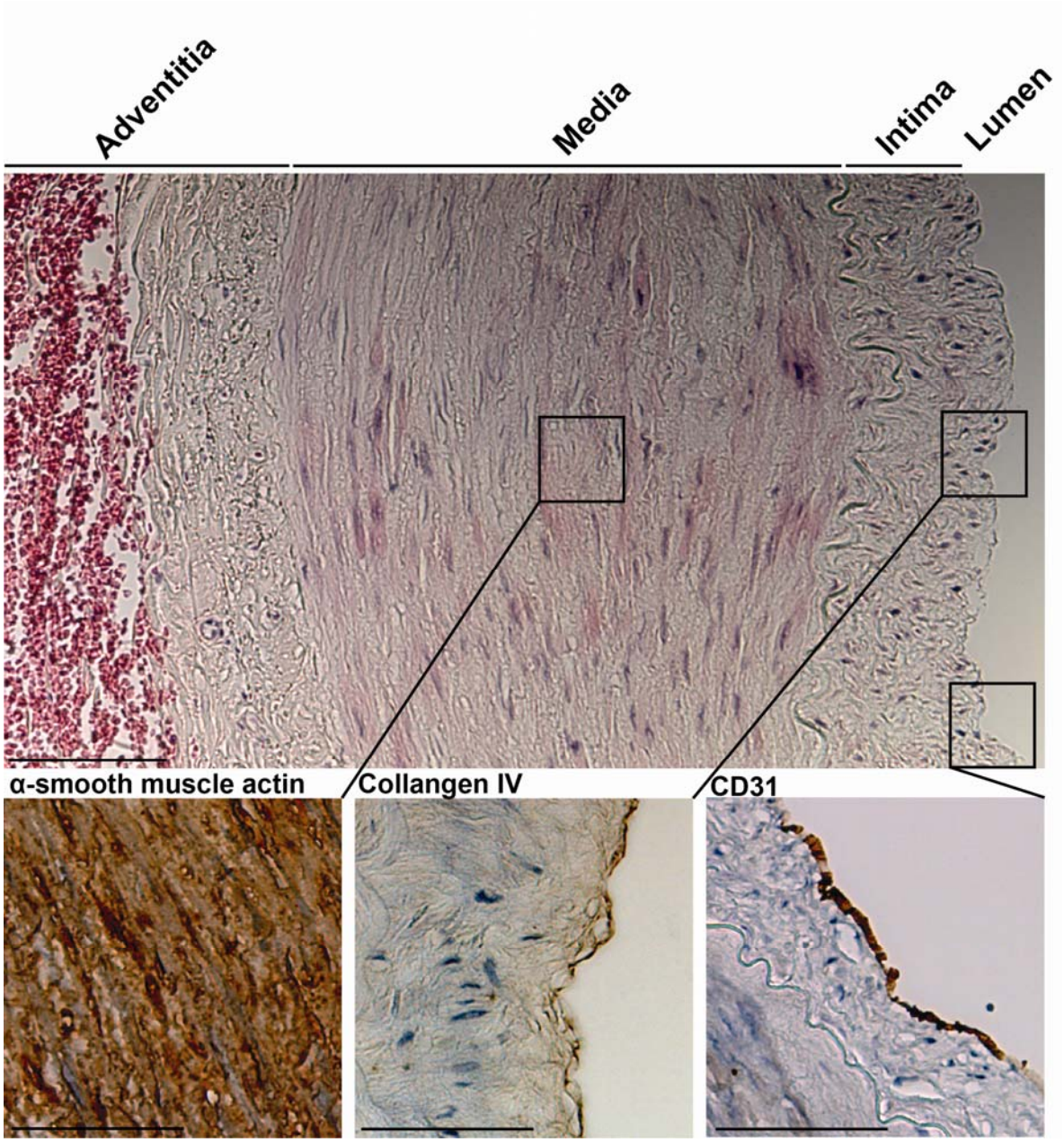

Figure 1: Architecture of a radial artery: *The three layer architecture is the adventitia, the media with specific staining against smooth muscle cells (α-smooth muscle actin) and the intima with specific stainings against the basement membrane (type IV collagen) and the endothelial cells (CD31).*

The development of atherosclerosis

Atherosclerosis is defined by the World Health Organization (WHO) as a complex pathological process of medium and large arteries that results in coronary artery disease (CAD) and cerebrovascular disease [3]. As emphasized by WHO atherosclerosis is an important public health problem and remains so far in the westernized countries the major cause of death.

Atherosclerotic lesions are characterized by the accumulation of lipids, fibrous elements and cells in the arterial wall over years. In the first decade of life, initial atherosclerotic lesions named “fatty streaks” develop. Although they are not clinically relevant they may involve into more complicated lesions [4]. Atherosclerotic lesions are caused by sustained high cholesterol level in particular in low density lipoproteins (LDLs) of the blood plasma [5]. With an increase of LDL plasma concentration, more LDL can insudate and accumulate within the arterial wall. The sites exposed to turbulent flow causing shear stress of the endothelium are preferential sites to accumulate LDL and further atherosclerotic plaque formation [6]. Whereas LDL is protected from modification in the plasma, it gets prompt for oxidation (oxLDL) within the arterial wall [5,7]. In addition to its direct cytotoxic properties to endothelial cells [8], oxLDL impairs the endothelial nitric oxide synthase (eNOS) expression and activity reducing vascular relaxation [9]. Moreover it induces the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and selectins on the surface of endothelial cells and it induces secretion of pro-inflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1) leading to monocyte recruitment into the arterial wall [10].

Monocytes accumulate within the arterial intima, differentiate into macrophages and take up modified LDL via scavenger receptors (SR-A and CD36) [11]. These two receptors unlike the LDL-receptor (LDLr) are not regulated by the cellular concentration of cholesterol. Therefore macrophages accumulate enormous quantities of cholesterol esters and differentiate into foam cells (Fig. 2) [5].

In addition to monocytes, lymphocyte T cells trans-migrate into the arterial wall and induce inflammation of the tissue [12]. Indeed a general inflammatory state is observed in

patients with atherosclerosis and several pro-inflammatory cytokines are detected within the plaque, among them the tumor necrosis factor α (TNFα), the interleukins (IL): IL-1β, IL-6, IL-8, the interferon γ (IFNγ), etc. (review in [13,14]). The variety of pro-atherogenic molecules including growth factors and cytokines secreted by the macrophage foam cells, the T cells and the endothelial cells are responsible of the migration of smooth muscle cells into the intima. Smooth muscle cells proliferate and produce extracellular matrix protein leading to the thickening of the arterial wall and to the development of a fibrous cap covering the lesion area [5].

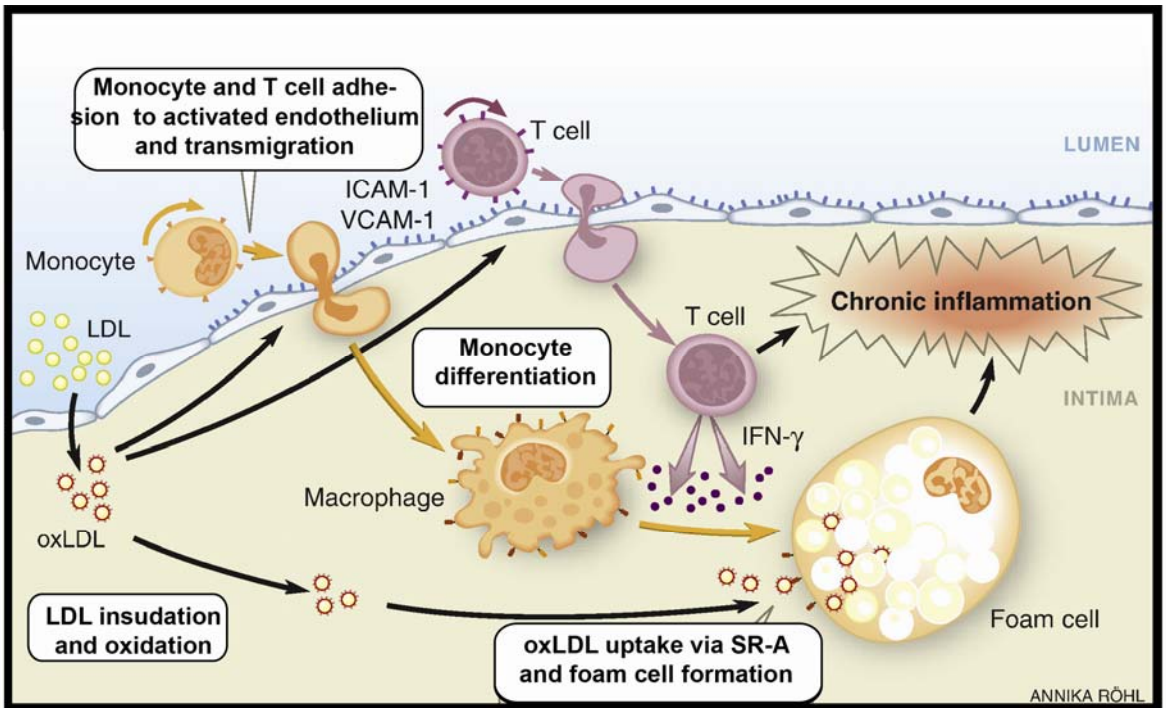

Figure 2: Initiating events in the development of atherosclerosis lesion. *LDL accumulates in the arterial wall and gets modified inducing endothelial cell activation. Monocytes and lymphocyte T cells adhere and migrate into the sub-endothelial space. Monocytes differentiate into macrophage and take up modified LDL via scavenger receptors leading to foam cell formation. T cells and macrophages foam cells secret inflammatory cytokines causing chronic inflammation of the tissue. Figure modified from Andersson et al.* [131].

Pathological studies suggest that the blood flow occlusion occurring during myocardial infarction or stroke is not caused by severe stenosis but rather by plaque rupture resulting in thrombosis. Vulnerable plaques are characterized by thin fibrous caps and increased number of immunocytes. Indeed foam cells secrete matrix metalloproteinases that destabilize the plaque by degrading extracellular matrix [15]. After plaque rupture the

exposure of the plaque content to the blood induces platelet aggregation and coagulation leading to blood clot formation and eventually to blood flow occlusion.

Cholesterol metabolism

Cholesterol plays a central role in the pathogenesis of atherosclerosis. Francois Poulletier de la Salle identified it first in 1769 in its solid form in gallstones however it was first named by Eugène Chevreul only in 1815. The name originates from the Greek names *chole* (bile) and *steros* (solid) followed by the chemical suffix *–ol* designating an alcohol. Cholesterol belongs to the class of steroid alcohol (sterol) and is a major constituent of the cellular plasma membrane in vertebrates where it regulates plasma membrane fluidity and permeability. In addition cholesterol is essential in humans for the synthesis of bile acids, steroid hormones and vitamins [16].

Cholesterol results from two sources, the *de novo* synthesis and the diet. The ratio between the *de novo* synthesis and the diet is about ~70:30. However this ratio probably varies considerably between individuals depending on diet and genetic dispositions [17]. Hepatocytes and enterocytes are the major source of the *de novo* synthesis even so all nucleated cells can synthesize it from the Acyl-CoA via the mevalonate pathway [18]. Statin drugs designed to reduce plasma cholesterol act on the limiting steps of this pathway and inhibit the HMG-CoA reductase to block the conversion of HMG-CoA to melavonate. Cholesterol synthesis by the cells is closely regulated by the transcription factor sterol-regulating element binding protein (SREBP). SREBP resides in the endoplasmic reticulum (ER) and in case of low cholesterol is transported to the Golgi where SREBP is further proteolytic processed [19]. The N-terminal fragment is then imported into the nucleus where it transcribes HMG-CoA reductase and the receptor LDLr genes to increase synthesis and up-take of cholesterol by the cell respectively [20]. Sufficient amounts of cellular sterols inhibit the ER-Golgi transport of SREBPs by interaction of the two chaperons SREBP cleavage activating protein (SCAP) and insulin induced gene (INSIG). Indeed when sterols bind to SCAP and INSIG the two chaperones bind together and trap SREBP in the ER [21]. Dietary cholesterol is taken up by the enterocytes and further transported to the liver.

Lipoprotein metabolism

Since cholesterol is a hydrophobic molecule, enterocytes and hepatocytes pack it into lipoproteins for the transport in blood. Lipoproteins are complex particles of lipids bound to proteins characterized by a shelf of phospholipids and cholesterol with at least one apolipoprotein and a core with triglyceride and cholesterol esters. Lipoproteins are differentiated according to their density (table 1). The density of the lipoproteins is related to the ratio between lipids and apolipoproteins. The less proteins the lower is the density.

Table 1: Lipoproteins classification

NAME	Density (g/ml)	Diameter (nm)	% proteins	Apolipoproteins
Chylomicron	< 0.95	100-1000	< 2	B_{48}, A-I, A-II, E, C-I C-II C-III, H
VLDL	0.95-1-006	30-80	10	B_{100}, C-I, C-II, C-III, E
IDL	1.006-1.019	25-50	18	B_{100}, C-II, C-III, E
LDL	1.019-1.063	18-28	25	B_{100}
HDL	> 1.063	5-15	33	A-I, A-II, A-IV, C-I, C-II, C-III, C-IV, D, E, F, H, J, L-I, M

Dietary lipids transport

Dietary lipids are absorbed by the small intestine particularly by the enterocytes after they were hydrolyzed by lipases. The absorbed lipolytic products -monoacylglycerol, free cholesterol, lysophospholipids and fatty acids- are resynthesized to phosphatidylcholines, triacylglycerol and cholesteryl esters and then assembled with apoB_{48} to chylomicrons that are further processed to the Golgi and secreted into the lymph [22,23]. In the circulation, chylomicrons exchange apolipoproteins (apoA-I, apoE and apoC-I) with the high density lipoprotein (HDL). In the capillaries of adipose tissue, skeletal and cardiac muscles, triacylglycerol of chylomicrons are hydrolyzed by lipoprotein lipase (LPL) to provide fatty acids and glycerol to adipocytes and myocytes. During this process chylomicrons size is reduced to become chylomicron remnants that are absorbed and further degraded by the liver through apoE receptor (apoEr) (Fig 3).

De novo lipids transport

The liver synthesizes triglycerides and cholesterol and packages them into the very low density lipoprotein (VLDL). The assembly of the VLDL begins by the lipidation of $ApoB_{100}$ in the ER by the microsomal triglyceride transfer protein (MTP) and its translocation from the ER lumen to the Golgi complex. MTP represent the limiting step in the formation of VLDL in the liver and chylomicron in the intestine, without this chaperon processing $apoB_{100}$ is blocked and degraded [24]. Nascent VLDL is released into the blood stream and maturates by exchanging cholesterol esters and triglycerides with HDL via the cholesteryl ester transfer protein (CETP).

VLDL triglycerides are hydrolyzed in the adipose tissues and musculature by the lipoprotein lipase (LPL) and converted into intermediated low density lipoprotein (IDL). IDLs are either taken up by the hepatic LDL receptor-related protein (LRP) or undergo hydrolysis of residual triglycerides by hepatic lipase (HL) (Fig 3).

The resulting LDL is composed of a single $apoB_{100}$ and in contrary to the VLDL, is a cholesterol ester rich particle. LDL is cleared from the plasma via the LDLr mostly in the liver.

The liver and the intestine produce one additional class of lipoproteins, HDL. ApoA-I, the main protein of HDL, is secreted by hepatocytes and the enterocytes in a ratio of 70:30 respectively. After secretion from hepatocytes and enterocytes, apoA-I acquires immediately phospholipids and cholesterol from the plasma mediated by the membrane ATP binding cassette transporter (ABC)A1 [25,26]. The resulting nascent HDL acquires additional cholesterol and phospholipids via ABCA1 and ABCG1 mediated efflux. Unesterified cholesterol transferred to HDL are esterified by the lecithin/cholesterol acyltransferase (LCAT). The cholesterol esters accumulate in the core and make HDL spherical. These particles are modified by a variety of enzymes. CETP swaps cholesterol esters from HDL for triglycerides from VLDL and LDL whereas the phospholipid transfer protein (PLTP) exchanges phospholipids and free cholesterol [27]. In addition HL and endothelial lipase (EL) remodel HDL by hydrolyzing triglycerides and phospholipids. HDL cholesterol is delivered back to the liver via interaction with SR-BI

which results in selective uptake of lipids or by holoparticle uptake through an as yet unknown pathway which is activated by ectopic β-ATPase (Fig. 3).

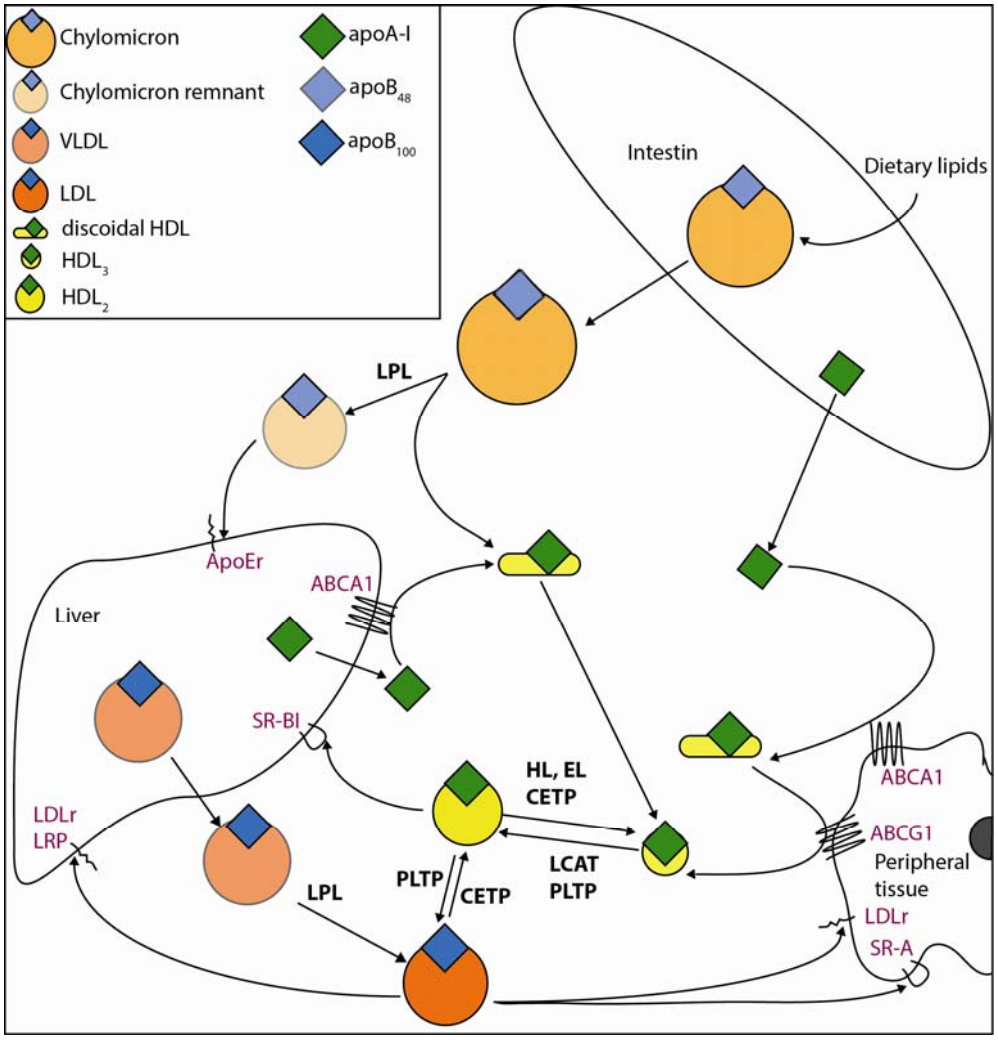

Figure 3: Lipoprotein metabolism. *The intestine absorbs dietary lipids and packages them with apoB$_{48}$ into chylomicrons, which are transported to peripheral tissues through the blood and then they are hydrolyzed by LPL. The chylomicron remnants are subsequently taken up by the liver by apoE receptor. De novo synthesized lipids are loaded onto apoB$_{100}$ particles to form VLDL which undergo lipolysis by LPL to form IDL and further LDL particles. IDL and LDL deliver cholesterol and phospholipids mainly to the liver through binding to the LDLr or LRP receptor. In atherosclerotic lesions LDL is modified and taken up through scavenger receptors which are not regulated by cellular cholesterol content. HDL is generated by the liver and intestine through the secretion of lipid-free apoA-I. ApoA-I then recruits cholesterol from these organs through the actions of ABCA1, to form discoidal HDL. In the peripheral tissues, lipid-free apoA-I and discoidal HDL promote the efflux of cholesterol through ABCA1 and ABCG1 respectively. The unesterified cholesterol in HDL is esterified to cholesteryl ester by LCAT, creating mature HDL. The HDL cholesterol is returned to the liver both directly, through selective uptake by SR-BI, and indirectly, by transfer to LDL through CETP. The lipid content of HDL is altered by the enzymes HL and EL and by the transfer proteins CETP and PLTP, affecting HDL catabolism (adapted from Rader et al.* [28]***).***

High density lipoprotein properties

HDL, often referred as the “good cholesterol”, is an heterogeneous class of lipoproteins, which are separated to distinct subclasses by different shapes, densities, sizes, lipid and protein contents [29]. HDL is produced as a small and lipid-poor (mainly phospholipids and cholesterol) discoidal shaped particle. LCAT activity converts HDL into spherical particle with a cholesteryl ester core, the predominating form in the plasma. Furthermore density classification differentiates two classes of HDL; a larger lipid-rich HDL_2 (1.063-1.125 g/ml) and a denser protein-rich HDL_3 (1.125-1.21 g/ml) [30]. A further classification based on the size further categorized HDL_2 and HDL_3 in five subpopulations of decreasing size from 10.6 to 7.6 nm: HDL2b, HDL2a, HDL3a, HDL3b and HDL3c [30].

Lipidome and proteome are also variable among the HDL particles. Currently lipidome analysis has revealed more than 200 individual lipid species in normolipidemic plasma, including cholesterol, triglycerides and phospholipids there are sphingolipids, phosphatidylcholines, ceramides, etc.[31]. In addition over 200 individual proteins have been identified and in these 85 were found by at least three independent laboratories and represent the best current estimation of the HDL proteome [32]. Interestingly recent finding revealed differences in the HDL proteome between healthy subjects and CAD patients some of them changing the properties of HDL [33]. Indeed HDL possess several anti-atherogenic properties inducing the improvement of vasculature function, the inhibition of vascular inflammation, the prevention of lipid oxidation, the inhibition of cell apoptosis and platelet aggregation as well as the stimulation of endothelial repair. Indeed the blood concentration of HDL-cholesterol is inversely correlated with the risk of cardiac events and strokes [34]. HDL is believed to remove cholesterol out of the macrophage foam cells in the atherosclerotic plaque and carried it back to the liver for disposal into the bile in a process named as reverse cholesterol transport (RCT) [35]. Cholesterol efflux from the macrophage foam cells to HDL, lipid-free or lipid-poor apoA-I was initially believed to be mediated by passive diffusion [36]. It is now clear that cholesterol efflux is facilitated by active transporters namely ABCA1 and ABCG1. ABCA1 mediates cholesterol efflux to lipid-free or lipid-poor apoA-I whereas ABCG1 mediates it to mature HDL [37].

Scavenger receptor BI

SR-BI is a 82-kDa membrane glycoprotein containing a large extracellular and two trans-membrane domains with a short cytoplasmic amino- and carboxyl-terminal domain [38]. SR-BI was initially found in the liver but is also expressed in other tissues and cells types such as macrophages and endothelial cells. SR-BI binds HDL with high affinity but also nevertheless other lipoproteins (LDL, oxLDL, etc.) [39]. The binding of HDL to SR-BI facilitates the uptake of cholesteryl esters into hepatocytes and steroidogenic cells in a process known as selective uptake [40]. The selective uptake to hepatocytes plays a central role in the RCT by removing cholesterol from HDL for excretion into the bile. Several lines of evidence suggest the central role of SR-BI in atherosclerosis. SR-BI$^{-/-}$ mice cross with atherosclerotic background (LDLR$^{-/-}$ or apoE$^{-/-}$) develop more atherosclerotic lesions [41]. Whereas mice with specific hepatic SR-BI overexpression demonstrate a decrease in atherosclerotic lesions [42]. Furthermore patients with a SR-BI mutation demonstrate an increased risk of cardiovascular events [43,44]. The role of SR-BI in cholesterol efflux has been intensively studied and demonstrated a minor role of SR-BI in promoting direct cholesterol efflux from macrophage foam cells in comparison to ABCA1 and ABCG1 [45]. Nevertheless SR-BI$^{-/-}$ bone marrow transplantation in apoE$^{-/-}$ mice demonstrated an increase of atherosclerosis lesions suggesting a protective role of macrophage SR-BI independent of cholesterol efflux [46].

In addition to its pivotal role in RCT, SR-BI regulates multiple processes in vascular cells important for vessel physiology. In endothelial cells the binding of HDL to SR-BI induces the activation of eNOS and the subsequent production of nitric oxide causing blood vessel relaxation [47]. The signaling cascade stimulating eNOS activity after HDL/SR-BI interaction has been identified and requires the capacity of SR-BI for cholesterol efflux, the C-terminal trans-membrane domain (CTTM) and the C-terminal cytoplasmic domain bound to the PDZ interacting protein. After cholesterol efflux, CTTM acts as a cholesterol sensor and PDZK1 initiates a kinase cascade involving SRC kinase, which activates Akt which phosphorylates eNOS at position 1177 [48].

SR/BI-HDL interaction also stimulates endothelial cell migration as well as endothelial repair and prevents of platelet activation and aggregation [49,50].

In conclusion SR-BI is both an important regulator of HDL cholesterol concentration in the blood and a multifunctional signaling HDL receptor.

Endothelial lipase

Endothelial lipase was first described in 1999 by two independent groups and is a phospholipase that belongs to the triacylglycerol lipase family including also LPL and HL [51,52]. EL is a 68-kDa glycoprotein sharing 44% and 41% amino acids sequence homology with HL and LPL, respectively. Nevertheless the lipolytic site largely differs between EL, HL and LPL. EL is mainly synthesized by vascular endothelial cells which gave its name however also other cells types such as macrophages and smooth muscle cells express it too [53]. EL is secreted into the extracellular space, where it binds to the cell surface heparan sulfates proteoglycans (HSPG) and exerts its lipolytic action [54]. Griffon et al. reported that EL forms a head-to-tail homodimer on the HSPG [55].

In contrast to LPL, which has mainly triglyceride lipase activity, and HL, which exhibits both triglyceride and phospholipase lipase activities, EL has a primarily sn-1-phospholipase activity and is a negative regulator of HDL cholesterol level. Over-expression of EL in mice demonstrated a marked decrease of HDL cholesterol and apoA-I concentration in the plasma due to an increase in the catabolic rate of HDL [56–58]. Concomitant with these findings, $EL^{-/-}$ mice or mice after pharmacological inhibition of EL results in bigger HDL particle size and an increase of HDL-cholesterol, apoA-I and phospholipid concentration in the plasma due to a decrease of the HDL catabolic rate [57,59,60].

Several rodent studies analyzed the role of EL in RCT but revealed contradictory results. Indeed Yancey et al. showed that mice overexpressing EL have smaller HDL particles and an increase of macrophage cholesterol efflux via ABCA1 but not ABCG1 [61]. In addition in vitro experiment showed that in macrophages, EL facilitates cholesterol efflux to apoA-I through its bridging function and lipolytic activity [62]. By contrast Brown et al.

revealed that the HDL from $EL^{-/-}$ mice increase cholesterol efflux compared to HDL from the wild type animals [60].

Although the role of EL in macrophage cholesterol efflux remains so far unclear it is consistently reported that EL increases the hepatic cholesterol uptake. Strauss et al. reported that EL mediates HDL binding and uptake in vitro via its lipolytic and bridging functions in hepatocytes and promotes the selective uptake of cholesterol esters [63]. Brown et al. reported that selective uptake is diminished in $EL^{-/-}$ mice [60]. Additionally the selective cholesterol uptake is enhanced in EL overexpressing mice dependently of SR-BI expression [56,64]. These findings indicate that the selective cholesterol uptake by SR-BI requires remodeling of HDL by EL.

The role of EL in the pathogenesis of atherosclerosis is not yet clear. Hirita and colleagues revealed that $EL^{-/-}$ mice have reduced atherosclerotic lesion development, whereas mice overexpressing EL have larger lesions [57,65]. Conversely Ko et al. reported no change in atherosclerotic plaque formation in $EL^{-/-}$ mice with $apoE^{-/-}$ and $LDLR^{-/-}$ background [66].

Several point mutations have been described in humans. The two low frequency mutations A396S and G26S impair lipolytic function and lower EL concentration respectively and are associated with a high HDL concentration in the plasma [67,68]. Another more common mutation T111I demonstrated no change in lipolytic activity compared to wild type EL and failed to correlate with HDL-cholesterol plasma concentration in a meta-analysis of five cohort studies [67]. The association of T111I with atherosclerosis is also controversial. Whereas two studies reported a decrease of CAD risk particularly in Chinese population [69,70], larger studies on Caucasian population revealed no association between the point mutation T111I and CAD [71–74]. Finally Cai et al. revealed that the point mutation A384C increased HDL and apoA-I blood concentration and reduced CAD risk in Chinese population [75].

In conclusion EL appears to have a central role in HDL metabolism. However its exact role in atherosclerosis genesis has to be further investigated.

ATP binding cassette

Some members of the ATP binding cassette (ABC) superfamily are regulating different steps in cellular cholesterol metabolism. Their functions are opposed to the LDLr and allow cells to export cholesterol and phospholipids. In humans the ABC superfamily comprises 48 transporters divided into 7 subfamilies from A to G [76]. Structurally the ABC transporters are distinguished into full transporters made of two units covalently bound and half transporters of single units forming of homo- or heterodimers. Each units of an ABC transporter is composed of six-trans-membrane α-helixes and an intracellular amino-terminal ATP binding domain also known as nucleotide binding domain (NBD). Among the full superfamily of ABC transporters at least four are believed to be involved in lipoprotein metabolism namely ABCG1, ABCG4, ABCG8 and ABCA1 [77]. As floppases they transport the lipids from the inner to the outer leaflet of membranes for excretion [78]. The role of ABCG1 and ABCA1 on HDL metabolism is further discussed in the following paragraphs.

ABCA1

ABCA1 is a full trans-membrane transporter of 240-kDA comprising two analogue structural units [79]. ABCA1 is predominantly expressed in hepatocytes, macrophages and astrocytes but other cells types such endothelial cells also express it.

The importance of ABCA1 in cholesterol metabolism was first highlighted in patients suffering from Tangier disease. These patients are characterized by a dramatic reduction of HDL particle concentration in the plasma as well as cholesterol ester deposition in various tissues specially the tonsils. An increase of lipid-free apoA-I catabolism causes the reduction of HDL-cholesterol concentration. Tangier disease is caused by different mutations in the ABCA1 gene preventing correct protein folding or expression in the hepatocytes [80]. ABCA1$^{-/-}$ mice show a similar phenotype as Tangier disease patients. Mice overexpressing ABCA1 have an elevated HDL concentration [81].

ABCA1 expression is enhanced by a high cholesterol content of the cells and is regulated by the nuclear receptor liver X receptor (LXR) [82]. ABCA1 is responsible of the transport of phospholipids and cholesterol to the external leaflet of the plasma

membrane where it is accessible to lipid-free or lipid-poor apoA-I but not to mature HDL. This process is best explained in hepatocytes and cholesterol loaded macrophages. Our group recently demonstrated that in endothelial cells ABCA1 also lipidates apoA-I [83].

ABCA1 is found in the intracellular compartment and on the cell surface and the location for apoA-I lipidation remains controversial. A first theory suggests that ABCA1 translocates cholesterol and phospholipids from the inner to the outer membrane leaflet by forming a transport chamber with its two trans-membrane domain. Cholesterol and phospholipids then form a lipid domain on the plasma membrane accessible for solubilisation by apoA-I [84,85]. A second model call the retro-endocytosis theory suggests that apoA-I binds to ABCA1 and the complex is internalized to intracellular lipids deposits where lipidation occurs before exocytosis [86,87]. Newer studies by Chen et al. revealed evidences that both mechanisms take place in the same cell [88].

The role of ABCA1 in the development of atherosclerosis has been intensively studied. The association of Tangier disease with CAD has been observed in some patients but not in other [89–91]. Animal studies revealed also controversial results. Aiello et al. demonstrated that ABCA1$^{-/-}$ mice or mice with ABCA1$^{-/-}$ bone marrow transplantation have increase atherosclerotic lesions [92]. However several studies reported that despite a marked changes in the plasma lipoprotein profiles and macrophage efflux, ABCA1$^{-/-}$ mice do not have increased atherosclerosis [81,93,94]. In addition, several studies with mice overexpressing ABCA1 demonstrated a reduction of atherosclerosis genesis [95–97]. Whereas Joyce et al. reported a significant increase in atherosclerosis in mice overexpressing ABCA1 [98]. The combine results of studies in Tangier disease patients, ABCA1$^{-/-}$ mice and mice overexpressing ABCA1 indicate that the effects of ABCA1 on genesis of atherosclerosis are complex and need further investigations.

Taking all together ABCA1 appears to play a central role in HDL cholesterol metabolism. However the exact mechanisms of apoA-I lipidation and cholesterol efflux remain so far unclear.

ABCG1

ABCG1 is a half transporter believed to be functional either as homo- or heterodimers with ABCG4 [99]. ABCG1 is predominantly expressed in hepatocytes and macrophages but also in other cells types.

Venkateswaran et al. revealed an induction of ABCG1 by the cholesterol signaling LXR-dependent nuclear receptor system and was the first to suggest its role in cholesterol metabolism [100]. In macrophages filled with cholesterol, LXR stimulates ABCG1 expression and its translocation from the intracellular compartments to the cell surface [101]. Suppression of ABCG1 in macrophages reduces the cholesterol efflux to HDL and further confirmed its role in cholesterol metabolism [102]. In addition ABCG1$^{-/-}$ mice have a marked accumulation of lipids in the tissues [103].

In contrast to ABCA1, which transports both phospholipids and cholesterol, ABCG1 is mainly a cholesterol transporter. After transporting cholesterol to the cell surface ABCG1 mediates cholesterol efflux from the cells to HDL but not to lipid-free apoA-I [85]. To corroborate the importance of ABCG1 in cholesterol metabolism Wiersma et al. revealed that ABCG1$^{-/-}$ mice have a decreased HDL level in the plasma and an increased cholesterol excretion into the bile [104]. However Kennedy et al. revealed no change in plasma lipids in ABCG1$^{-/-}$ mice but an accumulation in the tissues [103].

Whereas its role in macrophage cholesterol efflux is well documented, the function of ABCG1 in the development of atherosclerosis remains poorly understood. Several independent studies revealed an anti-atherosclerotic effect of ABCG1. Indeed ABCG1$^{-/-}$ mice or mice after ABCG1$^{-/-}$ bone marrow transplantation fed with high fat diet revealed an increase of atherosclerotic plaque development [105–108]. Nevertheless other studies demonstrated that ABCG1$^{-/-}$ mice or mice after ABCG1$^{-/-}$ bone marrow transplantation have reduced atherosclerotic lesions either in apoE$^{-/-}$ or LDLR$^{-/-}$ background [109–111]. In a more recent manuscript, Meurs et al. suggest that the differences of these studies may be due to the stage of the atherosclerotic lesions [112]. Moreover ABCG1 overexpression in the arterial wall reduces atherosclerotic lesion in rabbits [113].

In summary ABCG1 is an important key player in cholesterol efflux and HDL metabolism, however its exact role on atherosclerosis development needs to be further investigated.

Ectopic β-chain of the F0F1-ATPase

Martinez et al. identified for the first time in 2003 that the ectopic F_0F_1-ATPase binds apoA-I in hepatocytes [114]. Our group recently demonstrated that β-chain of the F_0F_1-ATPase binds apoA-I in endothelial cells [115].

The F_0F_1-ATPase is an enzymatic complex present on the membrane of prokaryotes, chloroplasts and mitochondria involves in the synthesis or hydrolysis of ATP. The F_0F_1-ATPase is composed of two units; the trans-membrane F_0 domain, which function as a proton channel [116] and the peripheral F_1 domain, which carries the catalytic site for ATP synthesis or hydrolysis [117]. Interestingly the F_0F_1-ATPase was found on the plasma membrane of endothelial cells, hepatocytes and tumor cells by cell surface biotinylation and confocal microscopy [114,118,119]. On the plasma membrane the F_1 domain is extracellular and fully functional. Although the mechanisms leading to the ectopic localization remains unknown, Wang et al. revealed that cholesterol loading induces its ectopic localization suggesting a possible role in cholesterol metabolism [120].

In addition to its mitochondrial function in ATP metabolism, the ectopic ATPase was reported to be a receptor for several ligands such as peptides, angiostatin and apoA-I [121]. Moreover in hepatocytes and endothelial cells apoA-I bound to the ectopic ATPase triggers HDL endocytosis [114,115]. Indeed upon binding of apoA-I, the F_0F_1-ATPase hydrolyses ATP to ADP, which activates the purinergic receptor $P2Y_{13}$ in hepatocytes and $P2Y_{12}$ in endothelial cells respectively. Activation of these receptors leads to G protein activations and increases HDL uptake [114,115].

In summary the β-chain of the F_0F_1-ATPase appears to plays a role in HDL endocytosis by endothelial cells and hepatocytes however its role in cholesterol metabolism and atherosclerosis genesis needs further investigation.

Trans-endothelial transport of HDL and apoA-I

Taking all together, RCT by HDL or by lipid-free apoA-I are both crucial in preventing atherosclerosis. However cholesterol efflux is taking place in the arterial wall therefore HDL and lipid-free apoA-I has to pass the endothelium barrier to get access to the cholesterol load macrophages. Several translocation routes are theoretically possible, diffusion through lesions, intercellular, active paracellular or intracellular transports.

Endothelial cell-to-cell junctions are characterized by the presence of tight junctions, adherens junctions and gap junctions and their main function is to limit paracellular permeability [122]. Therefore paracellular transport requires the opening of these junctions. This process is highly regulated by specific extrinsic and intrinsic signaling pathways and requires actin cytoskeleton remodeling [123]. Interestingly sphingosine-1-phosphate (S1P) stabilizes the integrity of endothelial barrier [123,124]. Since HDL carries more than 50 % of S1P [125] and it is a regulator of endothelium integrity [126], one may speculate that HDL is not transported paracellularly but rather intracellulary.

The intracellular transport or transcytosis is characterized by endocytosis, vesicular transport and exocytosis. This happens either receptor-independent (fluid phase) or receptor-mediated involving either clathrin or caveolin coated pits [123]. Chao et al. reported that fluorescent- and gold-labeled HDL appears to colocalize with caveolin in aortic endothelial cells [127]. In addition internalized HDL is found in vesicles in endothelial cells [128]. Our group demonstrated with aortic endothelial cells cultivated on a transwell system that HDL or lipid free apoA-I are transported through a confluent monolayer in a temperature dependent process [128,129]. Furthermore we found that ABCG1, SR-BI are involved in the transport of HDL while lipid-free apoA-I translocation depends on ABCA1 and the ectopic β-ATPase (Fig. 4) [115,128,130].

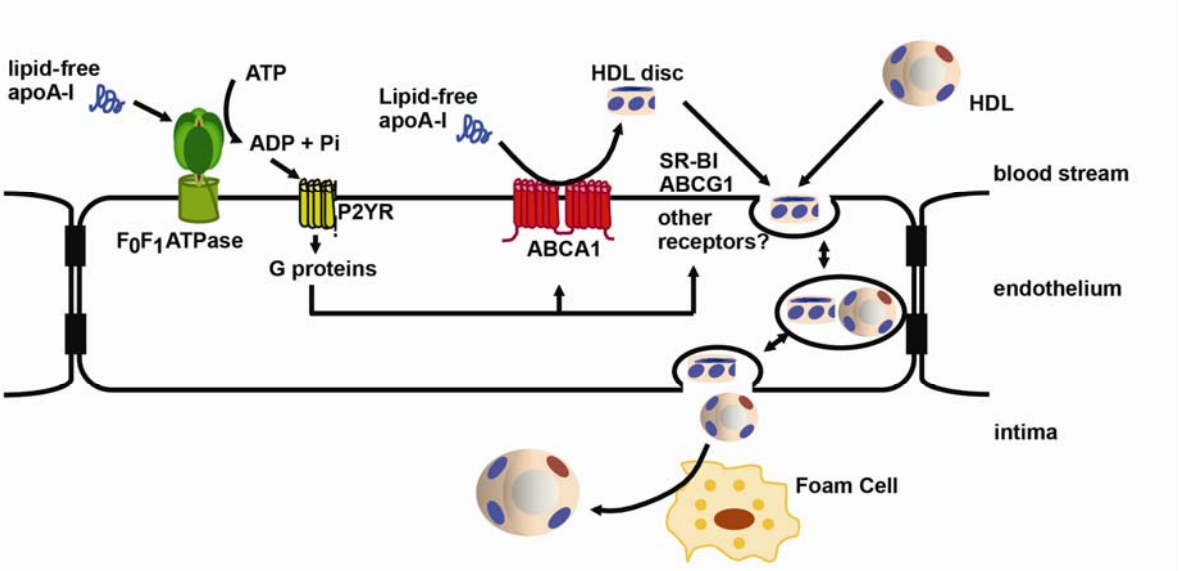

Figure 4: Trans-endothelial transport of HDL and apoA-I. *HDL binds to ECs and is internalized dependently of SR-BI and ABCG1. Lipids-free apoA-I binds to ABCA1 and gets lapidated. It is further internalized by ABCA1 independent mechanisms. In addition lipid-free apoA-I binds to the ectopic β-ATPase and signals via P2Y receptor to induce internalization of lipidated apoA-I and HDL* [115].

Aim of the present study

Our group intensively studied the trans-endothelial transport of HDL and apoA-I, however the inflammatory state of the atherosclerotic plaques may influence the transport through the endothelium. Therefore the aim of the present study is to analyze the transport of HDL and apoA-I through a monolayer of endothelial cells in the presence of inflammatory cytokines.

In contrast to the in vitro model, the native artery is a complex tissue composed of smooth muscle cells, endothelial cells and extra-cellular matrix. Therefore a second aim is to bioengineer an artery equivalent model. With this native like tissue-engineered artery model there will be many studies possible as the development of atherosclerotic plaque as well as the trans-endothelial transport of HDL under flow conditions ex vivo.

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Chapter

2

Interleukin 6 stimulates endothelial binding and transport of HDL through induction of endothelial lipase

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Short title: IL-6 stimulates endothelial transport of HDL

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Abstract

Objective: In the reverse cholesterol transport pathway, high-density lipoprotein (HDL) passes the endothelial cell barrier by mechanisms involving the scavenger receptor (SR)-BI and the ATP binding cassette (ATP) G1. However little is known on how inflammation influences this trans-endothelium transport. We hypothesize that interleukin (IL)-6 influences the trans-endothelial transport of HDL.

Approach and results: Upon stimulation with IL-6 cultivated primary endothelial cells showed increased binding and transport of ^{125}I-HDL without changing the expression of SR-BI and ABCG1. Therefore we analyzed the involvement of endothelial lipase (EL), a known HDL binding protein expressed by endothelial cells. Here we show an increase of EL expression after IL-6 stimulation. Moreover using pharmacological inhibitors or RNA interference against EL we demonstrated its participation in HDL binding and transport through the endothelium. EL was also found responsible for the reduction of HDL-particle size occurring during the specific transport through a monolayer of endothelial cells. Finally, pharmacological inhibition of EL after IL-6 stimulation reversed the inducing effect of IL-6 on HDL binding and transport.

Conclusion: IL-6 stimulates the translocation of HDL through the endothelium, the first step in reverse cholesterol transport pathway.

Introduction

Atherosclerosis is a chronic disease characterized by lipid retention and inflammation in the arterial wall. Many studies revealed that the cholesterol concentration of plasma high density lipoproteins (HDL) is inversely correlated with the risk of coronary artery disease events [1]. The cardio protective role of HDL is, in part, related to its ability to remove the cholesterol from the macrophage foam cells in the arterial wall and carry it to the liver for excretion into the bile [2]. An early step in the reverse cholesterol transport is the transfer of cholesterol from the macrophages to the HDLs. Importantly the loading of cholesterol is not taking place in the plasma but in the arterial intima [2]. Consequently HDL has to cross the endothelium barrier to get access to the cholesterol-loaded macrophages. We previously demonstrated that endothelial cells bind, internalize and transcytose HDL in a saturable and temperature dependent manner. Using siRNA and pharmacological interferences we demonstrated that endothelial cells bind and transport HDL by distinct specific mechanisms involving the scavenger receptor (SR̃)BI and the ATP binding cassette transporter (ABC) G1 but not ABCA1 [3].

In inflammatory diseases such as atherosclerosis, many (inflammatory) cytokines have been demonstrated to be elevated in patient plasma among them the tumor necrosis factor α (TNFα), the interleukins (IL) -1β and IL-6 [4]. Moreover Hingorani and Casas as well as Sarwar et al. recently demonstrated a correlation between the IL-6 receptor pathway and atherosclerosis [5,6]. IL-6 has been also demonstrated to increase the cholesterol efflux to HDL in macrophages [7]. Consequently we hypothesized that IL-6 may modulate the trans-endothelial transport of HDL. Indeed, we provide evidences that IL-6 enhances binding and transport of HDL in an SR-BI and ABCG1 independent way. This observation raised the possibility that an additional protein is involved in the binding, internalization and transport of HDL through endothelial cells. In a candidate based approach we investigated if endothelial lipase (EL) is involved. Vascular endothelial cells express and secrete EL a glycoprotein of 68-kDA which binds to the cellular proteoglycans where it exerts its action, EL binds HDL, hydrolysis HDL-phospholipids at the sn-1 position and is a regulator of the plasma HDL cholesterol level [8,9]. Moreover, several groups demonstrated a positive correlation between the expression of EL and inflammation in vitro as well as in vivo [10–13]. In this study, we

provide evidences that EL participates in HDL binding, cell association and transport. In addition IL-6 induces EL expression in endothelial cells and consequently the HDL binding and transport is enhanced.

Material and Methods

Endothelial binding, cell association and transport of HDL

The methods for the isolation and radiolabeling of HDL, the cultivation of bovine aortic endothelial cells as well as the quantification of endothelial binding, association, internalization, and transport of HDL have been described previously [3,14,15]. In brief, endothelial cells were incubated with 10 μg/ml of ^{125}I-HDL without (total) or with a 40 folds excess of non-labeled HDL (non-specific) for 1 hour at 4 °C for the cell binding and 37 °C for the cell association and transport. Specific binding/association/transport was calculated by subtracting the non-specific binding/association/transport to the total binding/association/transport. All experiment were done in at least triplicate.

Pharmacological treatment and inhibitors

Endothelial cells were incubated with IL-6 (Preprotech, USA) at a final concentration of 10 ng/ml for 24 h in DMEM containing 0.2% BSA prior the assay. To test the involvement of EL in HDL cell binding, association and transport endothelial cells were incubated with heparin (Sigma) at a final concentration 100 μg/ml of or with heparinase III (Sigma) at a final concentration of 0.2 U/ml for both 30min priors the assay. THL (Sigma) was added to the cells at a final concentration of 25 μM in DMEM hepes containing 0.2% BSA 30 min prior adding ^{125}I-HDL.

SiRNA transfection

Endothelial cells were transfected when 80% to 90% confluent. Sixty-seven nmol/L BLOCK-iT^{TM} fluorescent oligo and 100 nmol/L siRNA against EL (GCA AGC AUC CUC CUU GCU UTT, GCU CGG UGA GUU CGA CAA ATT or AUC CAU GUC UUC AGC UAC ATT) (Microsynth, Switzerland), SR-BI (TCG TCA TGC CCA ACA TCC TGG TCT T) (Invitrogen) or not coding siRNA were transfected with Lipofectamine 2000 in

OPTIMEM (Invitrogen) according to the manufacturer's protocol. Six hours after transfection, the medium was replaced by DMEM 5% FCS. Binding, cell association, transport and gel filtration assays were conducted between 60 and 72 hours after the transfection.

Quantitative real time PCR

Total RNA was isolated from the cells using RNA Bee (Ambio, Switzerland) according to the manufacturer's instruction. Genomic DNA was removed by digestion with DNase (Roche). Reverse transcription was performed using ReverstAid H Minus (Fermentas, Thermo Scientific) and following the standard procedure as defined by the manufacturer. Quantitative PCR was done with LightCycler FastStart DNA Master SYBR Green I (Roche). Genetic specific primers were used as followed: ABCG1 (for: GAG GAA GAA AGG ATA CAA GAC C; rev: GTC AGT ATC TCC TTG ACC ATT TCC), SR-BI (for: GGA ATC CCC ATG AAC TG; rev: CTT GGG AGC TGA TGT CAT C) and EL (for: CAC CAA CAC CTT CCT GGT CT; rev: TTT TTC ATC CTC CAG CCA TC) and compared to actin (for: TGC CCT GGC ACC CAG CAC AA; rev: AGG TGG ACA GCG AGG CCA GG). Conventional PCR was carried out using following primers: GAPDH (for: CCC ATG TTC GTC ATG GGT GT; rev: TGG TCA TGA GTC CTT CCA GCA TA).

Western Blotting

Endothelial cells were lysed in RIPA buffer (10 mmol/L Tris pH 7.4, 150 mmol/L NaCl. 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, complete EDTA (Roche)). Equal amounts of total protein were separated on a SDS-PAGE and trans blotted onto a PDVF membrane (GE Healthcare). The expression of ABCG1 (anti ABCG1 E20 Santa Cruz), SR-BI (anti SR-BI E4 Novus or anti SR-BI EP1556Y ABCAM) and EL (anti EL NB 400-118 Novus) were evaluated and compared to the expression of actin (anti actin AC-15, Sigma). Intensity of the blot was quantified using Image J.

Gel filtration chromatography

The size of HDL before and after transport studies through cells previously treated with THL or RNA interference against EL and SR-BI was analyzed by gel filtration chromatography as previously described [3].

Lipase activity assay

The lipase activity of endothelial cells was measured using PED-A1 (Invitrogen) following the manufacturer instruction as previously described by Darrow, A. et al. with the following modifications [9]. Endothelial cells (2.5 $E10^6$) after IL-6 or THL incubation were treated with heparinase as described previously in 0.9% NaCl. Supernatant was collected and concentrated 10 folds with 30K Amicon Ultra concentrator (Millipor) before incubating with PED-A1 for 2 hours.

Statistical analysis

The data for all experiments were analyzed using GraphPad Prism 5 software program. Comparisons between groups were performed using t-test or ANOVA. Experiments were routinely performed in triplicate. The data have been obtained from at least 3 different experiments and are graphically represent as mean ± standard deviation, if not indicated otherwise. n.s. not significant; * P<0.05; ** P<0.001; *** P<0.0001

Results

IL-6 induces HDL binding, cell association and transport

To investigate whether the interaction of HDL with endothelial cells is changed by the inflammatory cytokine IL-6, we stimulated aortic endothelial cells with IL-6. The interaction of HDL with the endothelial cells was characterized as binding at 4 °C, cell association and transport at 37 °C. After 24 hours of IL-6 stimulation, specific binding was induced by 200% compared to not stimulated cells (Fig. 1A). Conversely the specific cellular associated HDL and the transported HDL were also induced by 144% and 178% respectively (Fig. 1B and C). Moreover to rule out that the induction of HDL transport was due to permeability changes after IL-6 stimulation, the integrity of the endothelial cells monolayer was assessed by analyzing the permeability with ^{3}H-inulin. The tracer was added to the apical chamber and the radioactivity in the basolateral chamber was counted. The permeability coefficient was not changed after IL-6 stimulation compared to the control, namely 1.756 $E10^{-5}$ ± 0.156 $E10^{-5}$ cm/s and 1.863 $E10^{-5}$ ± 0.208 $E10^{-5}$ cm/s (Fig. 1D) over a time period of 1 hour.

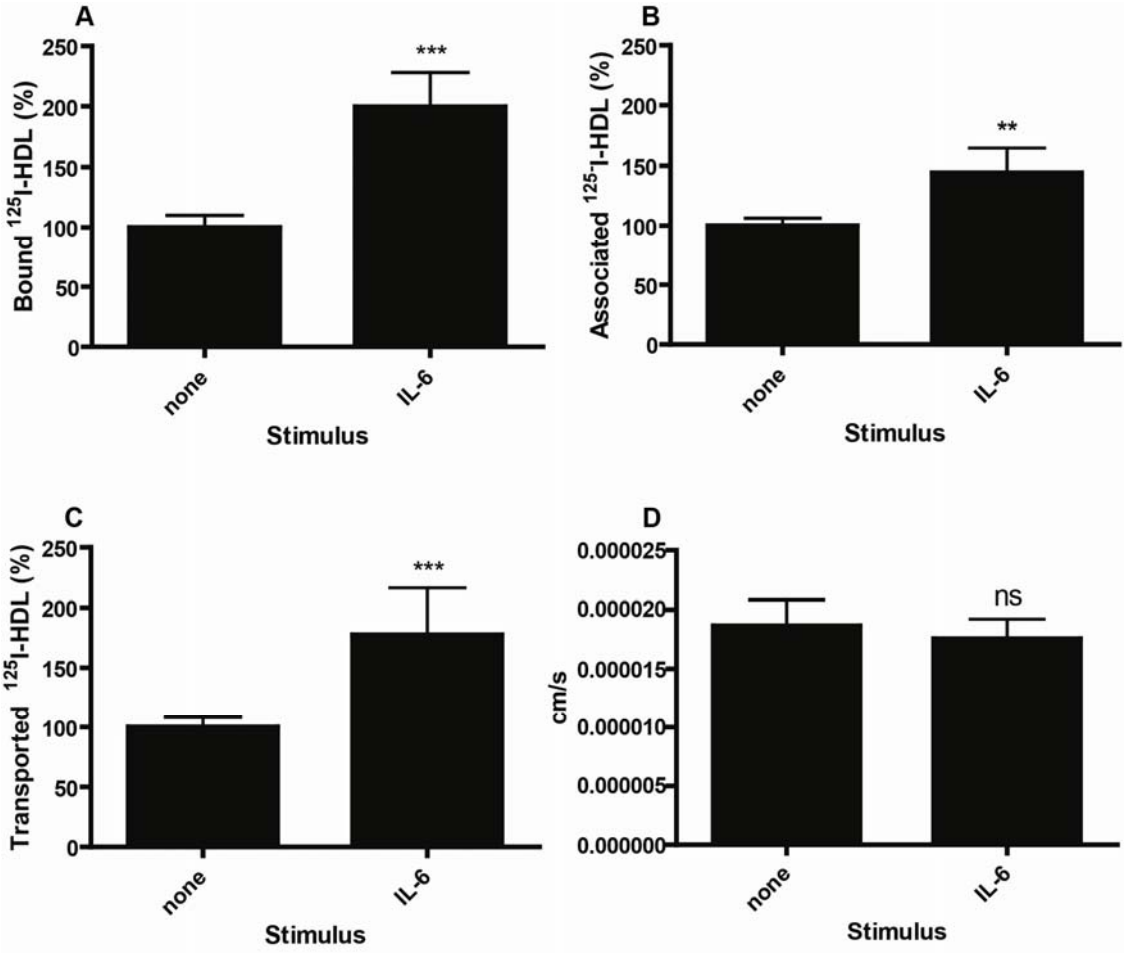

Figure 1: Influence of IL-6 on endothelial binding at 4°C (A), cell association at 37°C (B), and transport of HDL (C) as well as permeability (D). *ECs were stimulated with IL-6 (10 ng/ml) for 24 hours prior the assays. Binding (A) was measured by incubating the cells with 10 μg/ml ^{125}I-HDL at 4 °C. To analyze the association (B) and transport (C) ECs were cultivated for 24 hours on insert before IL-6 stimulation then ^{125}I-HDL was added to the apical compartment. After one hour at 37 °C cells were lysed and specific activity was counted to obtain cell association (B). Medium of the basal compartment was harvested and specific activity was monitored to obtain the transport (C). The permeability of the cell monolayer to inulin was determined (D). The results are represented as mean ± SD of at least 3 individual experiments made of triplicate.*

Previously, we demonstrated that ABCG1 and SR-BI modulate the endothelial cell interaction with HDL. Therefore, we analyzed the mRNA level and protein expression of ABCG1 and SR-BI after IL-6 stimulation. Interestingly, real time RT-PCR analysis did not demonstrate any changes in the expression levels of ABCG1 and SR-BI after IL-6 stimulation, namely 1.23 ± 0.33 fold for SR-BI and 1.10 ± 0.49 fold for ABCG1 relative

expression compared to not stimulated cells set to 1 (Fig. 2A & B). Moreover western blot analyses of the protein expression levels of ABCG1 and SR-BI after stimulation were not significantly changed (respectively 1.07 ± 0.44 fold and 0.89 ±0.37 fold) (Fig. 2C & D). These results indicated that enhanced cell binding, association and transport of HDL after IL-6 stimulation is independent of SR-BI and ABCG1.

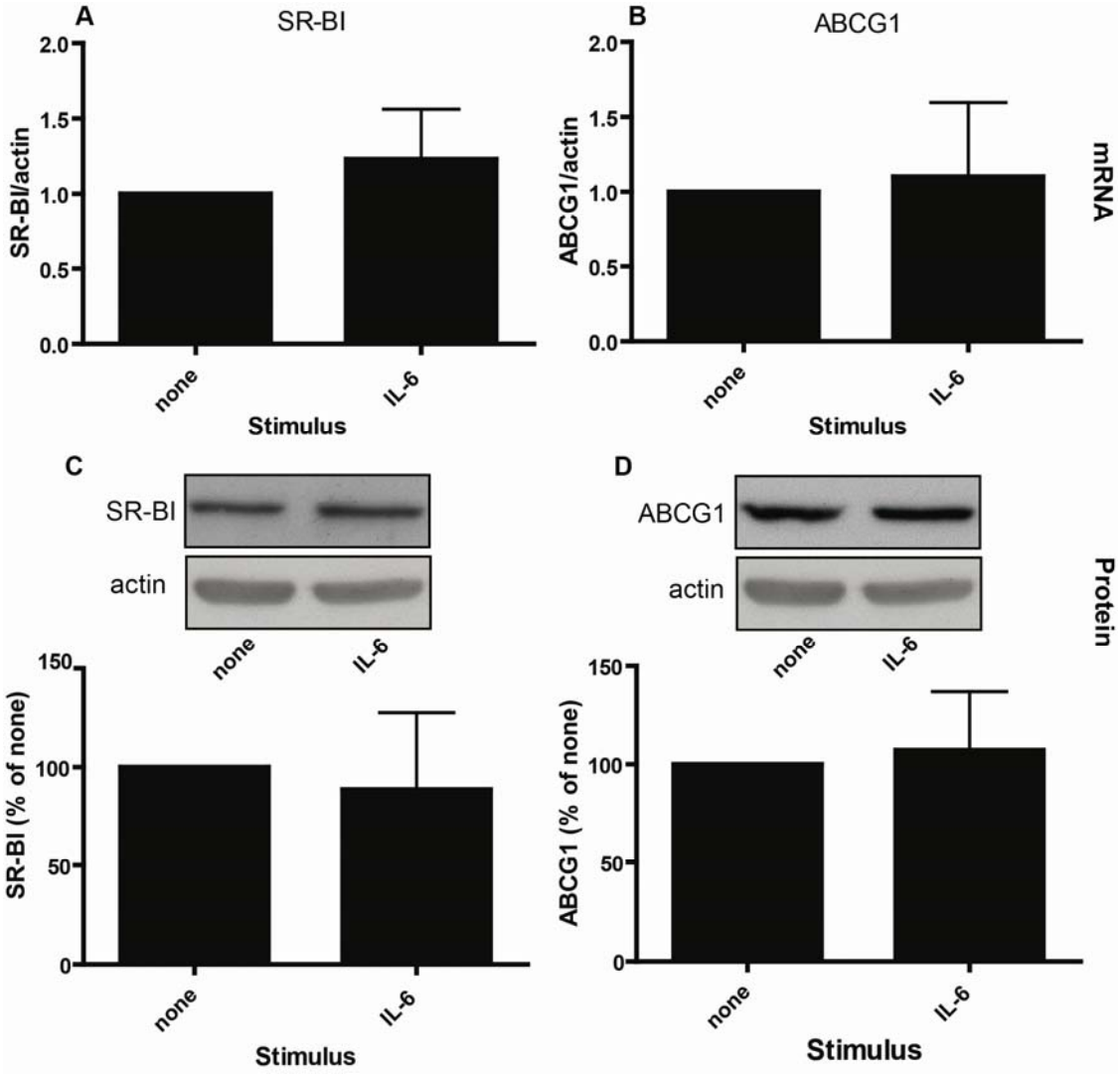

Figure 2: mRNA and protein expression of SR-BI (A, C) and ABCG1 (B, D) after IL-6 stimulation (10 ng/ml; 24 h). *The mRNA expressions of SR-BI (A) or ABCG1 (B) were quantified using real time RT-PCR and compared to the expression of actin. The protein levels of SR-BI (C) and ABCG1 (D) were evaluated by western blotting. Representative blots and quantifications of band intensities of at least 3 experiments are presented. The results are represented as mean ± SD of at least 3 individual experiments.*

Identification of EL modulating trans-endothelial transport

We hypothesized that EL mediates HDL binding, cell association and transport. First we confirmed the expression of EL in endothelial cells by PCR (Fig. 3A) and by western blot analysis (Fig. 3B). Then the effect of IL-6 stimulation on EL expression was analyzed. After 24h stimulation with IL-6, EL expression was doubled on both the RNA level (2.12 ± 0.41 fold) (Fig. 3C) and the protein level (1.98 ± 0.84 fold, Fig. 3D) as revealed by real-time PCR and western blotting analyses, respectively. To evaluate the role of EL in HDL binding we treated the cells with pharmacological inhibitors as well as by reducing its expression by RNA interference. Binding of HDL was reduced to 79 ± 10 % after treatment of the cells with heparin. Moreover after digesting the cell surface proteoglycan with heparinase, binding of HDL was reduced to 65 ± 15 %. In addition treating the cells with the lipase inhibitor tetrahydrolipstatin (THL) reduced the binding to 53 ± 22% (Fig. 4 A). Finally specific RNA interference against EL reduced binding, cell association and transport to 52 ± 12%, 71 ± 14 % and 47 ± 8%, respectively (Fig. 4 B-D). On the transcriptional level siRNA treatment revealed a decrease to 10 % on mRNA expression (data not shown) and to ≅ 50 % on protein expression compared to non-coding siRNA transfected cells whereas the expression of SR-BI and ABCG1 remained unchanged (Fig. 4F).

EL enhancement after IL-6 causes increase of HDL binding and transport

To analyze the role of IL-6 in EL expression and consequently HDL binding and transport, the endothelial cells were stimulated with IL-6 and treated with THL to block the HDL binding to EL. The IL-6 stimulation enhanced the binding and transport of HDL to 140 ± 20% and 184 ± 30 % respectively. However THL treatment after IL-6 stimulation reduced the binding by 65 ± 17% and transport by 77 ± 30% compared to IL-6 stimulation and compared to the not stimulated cells by ≅ 50 % for both binding and transport. The difference between the THL treated cell with or without IL-6 was not significantly different for the binding and transport of HDL (Fig. 5 A and B). Moreover, IL-6 stimulation increased the lipase activity of 130 ± 6% in the endothelial cells (Fig. 5C).

To verify that the obtained activity was EL dependent the cells were transfected with specific siRNA against EL and resulted in a reduction off 43 ± 30% in lipase activity (data not shown). Moreover endothelial cells treated with THL reduced the lipase activity by ≅ 50 % (Fig. 5C). These results indicated that the up regulation of EL expression is responsible of the enhancement of cell binding and transport of HDL through endothelial cell after IL-6 stimulation.

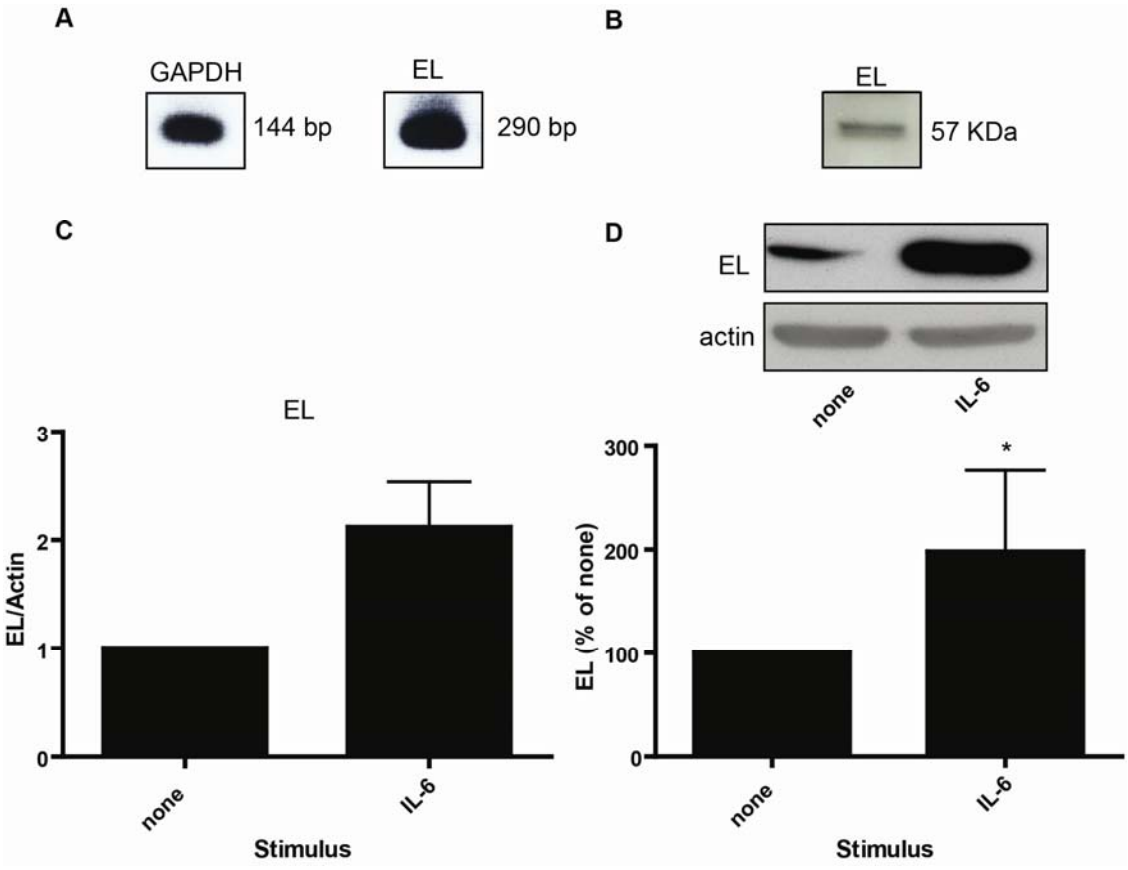

Figure 3: Role of IL-6 on the expression of endothelial lipase. *The basal expression of EL by endothelial cells was analyzed by PCR (A) or by western blotting (B). Effects of 24 hours stimulation with IL-6 on mRNA (C) and protein levels (D) of EL were analyzed by real time PCR or western blotting. The results are represented as means ± SD of at least 3 individual experiments.*

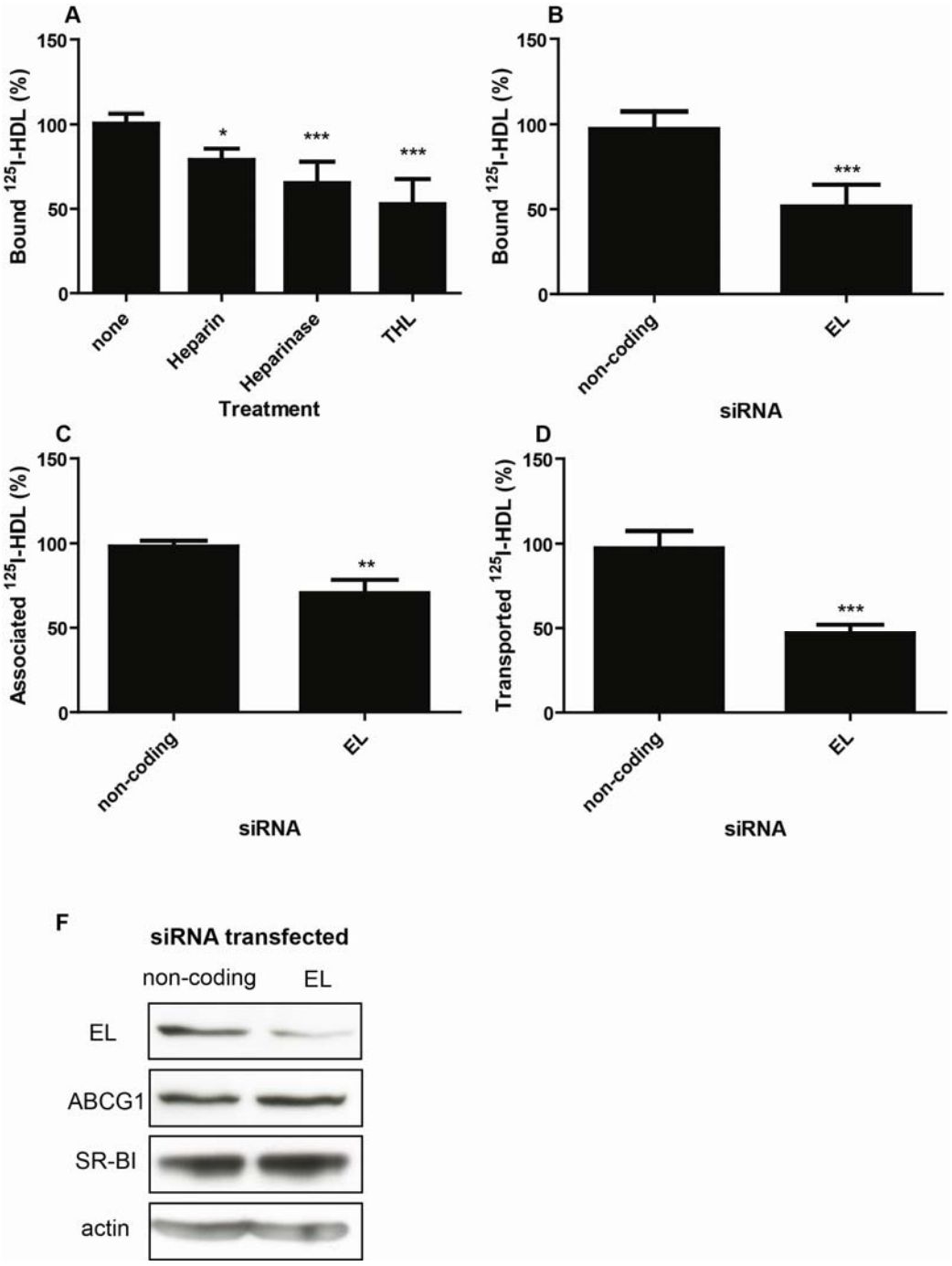

Figure 4: Role of EL in HDL binding (A, B), cell association (C) and transport (D). *To address the role of EL, pharmacological inhibitors (A) as well as specific siRNA for EL (B-D) were used. The binding of HDL was assayed after 30 min pre-incubation with heparin (100 μg/ml), heparinase (0.2 U/ml) or THL (25 μM) (A) or 72 hours after siRNA transfection (B). Cell association (C) and transport (D) were evaluated 72 hours after the transfection. Protein expression of EL, SR-BI or ABCG1 (E) was evaluated 72 hours after the transfection. The results are represented as mean ± SD of at least 3 individual experiments made of triplicates.*

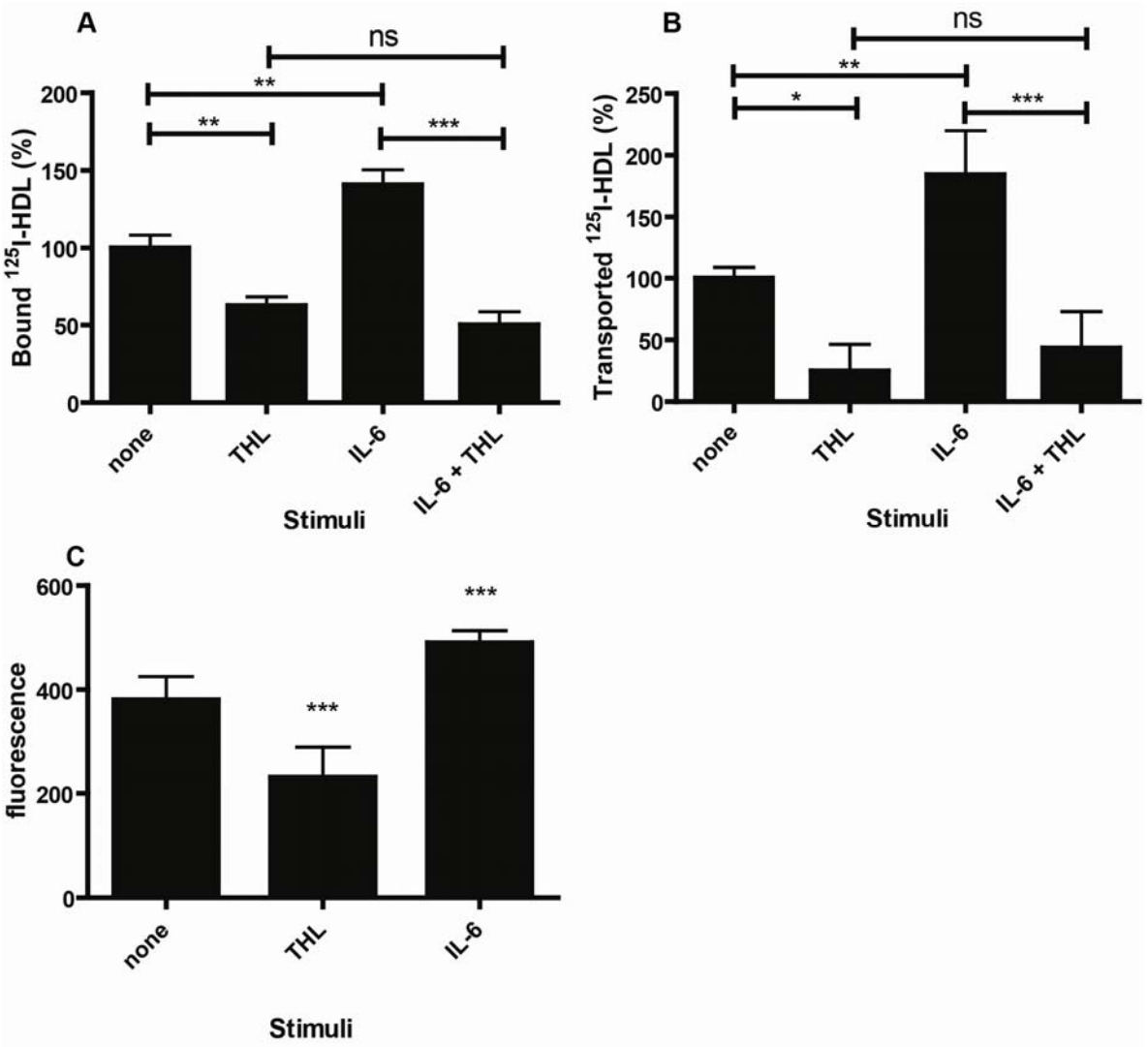

Figure 5 Role of lipolytic EL activity on the enhancement of HDL binding and transport after IL-6 stimulation. *Cells were incubated with or without IL-6 for 24 hours in the presence or absence of 10 µM THL, added 30 minutes prior to the assay. Binding (A) and transport (B) were performed as described earlier. The endothelial lipase activity of endothelial cells (C) was analyzed using PED-A1. The results are represented as mean ± SD of at least 3 individual experiments made of triplicates.*

EL is responsible of size reduction of HDL after cell contact.

We previously demonstrated that HDL particles transported through a monolayer of endothelial cells are recovered at reduced size. Gel filtration analysis of the transported material demonstrated a Stokes diameter of 9.74 ± 0.25 nm whereas the Stokes diameter of the starting HDL was of 10.63 ± 0.06 nm (Fig. 6). Interestingly, the protein moiety was not changed as previously demonstrated by SDS page analysis. To assess

if EL or SR-BI are responsible of the size reduction of HDL, we used pharmacological inhibitors as well as RNA interference against EL and SR-BI. After both interference with SR-BI siRNA and non-coding siRNA, trans-endothelial transport led to the recovery of smaller particles with Stokes diameter of 9.81 ± 0.01 nm and 10.01 ± 0.015 nm, respectively (Fig. 6). In contrast, after silencing of EL, the HDL particles that were recovered in the basolateral compartment were less prominently and not significantly reduced in size (Stoke diameter of 10.25 ± 0.12 vs. 10.63 ± 0.05 nm for HDL without cell contact, Fig. 6). To corroborate this finding we blocked the lipase activity of EL with THL and found the size reduction of HDL abolished as well (Fig. 6). These data suggested that EL but not SR-BI contributes to the particle size reduction of HDL during trans-endothelial transport.

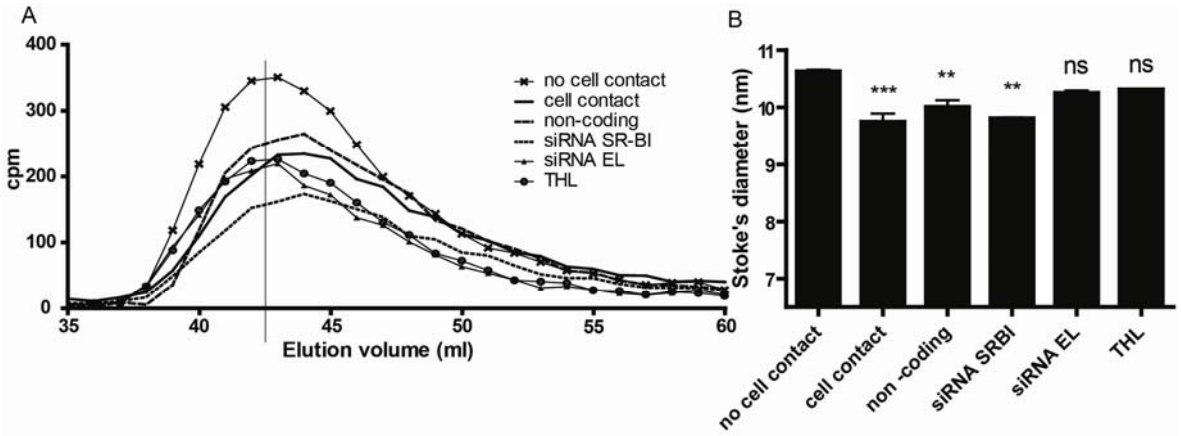

Figure 6: Effect of EL and SR-BI on the size reduction of HDL after cell contact. *A, endothelial cells were cultivated on inserts, ^{125}I-HDL was added to the apical chamber and incubated for one hour. HDL retrieved from the basolateral compartment after transport through ECs treated with indicated pharmacological inhibitors or siRNAs were analyzed by gel filtration chromatography and compare to HDL with cell contact. The curves represent average results of at least triplicate experiments (A). The bar diagrams represent means ± SEM of at least 3 individual experiments (B).*

Discussion

We previously reported that HDL is transported through a monolayer of cultivated endothelial cells in a specific process which involves SR-BI and ABGC1 [3]. Here, we assessed the question, if HDL binding, cell association and transport though the endothelial cells are modulated by the pro-inflammatory cytokine IL-6. IL-6 strongly correlates with the development of atherosclerosis [4–6] and its presence has been demonstrated in the atherosclerotic lesions [16].

Inflammatory cytokines have been demonstrated to modulate trans-endothelium transport. For example, Dohgu et al. demonstrated an increase in HIV transport after IL-6 stimulation whereas Wang et al. a reduction of insulin transport after IL-6 stimulation [17,18]. However to our knowledge, we demonstrate for the first time that IL-6 enhances the capacity of endothelial cells to bind, associate and transport HDL. Importantly inflammatory cytokines such as TNFα, IL-1β or IL-6 have been demonstrated to increase the permeability of the endothelial barrier in a concentration and time dependent manner [19–21]. Therefore in the present study, we ruled out that the concentration of IL-6 used, enhances transport by increasing the permeability of the endothelial barrier. Also unlike in human peripheral monocytes [22] IL-6 stimulation did not cause any changes in RNA or protein levels of SR-BI or ABCG1 in endothelial cells. These findings clearly indicate that the increased HDL binding and transport of HDL in IL-6 stimulated endothelial cells is not mediated by SR-BI or ABCG1 but by another protein. We hypothesized the involvement of endothelial lipase (EL) which is produced and secreted by endothelial cells to be subsequently bound by heparan sulphate proteoglycan (HSPG) in their extracellular matrix [23]. After confirming the expression of EL in the endothelial cells its role in HDL binding and cell association, was assed using pharmacological treatments and RNA interference.

Digestion of HSPG with heparinase to release the bound EL or competition for EL binding to HSPG with heparin reduced HDL binding on the cell surface. Moreover the reduction in HDL binding after blocking EL activity by the lipase inhibitor THL corroborates the involvement of EL in HDL binding. Interestingly, in hepatocytes it is reported that blocking EL by THL induces the binding of HDL [24]. The role of EL in the

binding of HDL was further confirmed by specific RNA interference against EL and showed a dramatic reduction in HDL binding. Specific knockdown of EL also demonstrated the involvement of EL for HDL internalization and transport of HDL trough the endothelial cells. Similarly specific knockdown of EL was previously shown to reduce binding and internalization of HDL into hepatocytes [24]. These results clearly indicate EL as an important mediator of HDL binding in endothelial cells as well as in hepatocytes. We previously demonstrated that the size of HDL particles decreases during trans-endothelial transport without degradation of the protein moiety [3]. Because of the involvement of SR-BI in trans-endothelial HDL transport one possible explanation was the extraction of cholesteryl esters by selective uptake. [25]. Alternatively lipolysis of phospholipids by EL [26] may also cause the remodeling of HDL during transport. Therefore we investigated if SR-BI, EL or both are responsible for the HDL particle size reduction in endothelial cells. Specific RNA interference experiments clearly showed that EL but not SR-BI is responsible for HDL particle size reduction. The finding was confirmed by blocking the lipolytic activity of EL with THL.

Several studies have shown that inflammation increases the level of EL on both endothelial cells and in the circulation [11,12,27]. Therefore in the present study we analyzed the role of EL in the interaction of HDL with endothelial cells after IL-6 stimulation. The endothelial cell binding, cell association and transport of HDL after IL-6 stimulation was enhanced due to an enhancement of EL activity caused by up regulation of EL expression. Blocking EL with THL reversed the IL-6 stimulated effect. These data clearly indicate that IL-6 stimulates the translocation of HDL through the endothelium the first step in reverse cholesterol transport pathway.

In conclusion our study demonstrated for the first time an induction of HDL binding, cell association and transport of HDL after IL-6 stimulation. By identifying EL as the endothelial target of IL6 stimulation we unraveled EL as a novel contributor to trans-endothelial HDL transport in addition to SR-BI and ABCG1.

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Disclosure

None.

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Chapter

3

The inflammatory cytokines, IL-1β and TNFα reduce apoA-I and HDL transport through endothelial cells

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Short title: IL-1β and TNFα reduce HDL and apoA-I transport through endothelial cells

Contribution JR:
Participated to the experimental design, performed most of the analyses and wrote the manuscript

Abstract

Objective: In the reverse cholesterol transport pathway, high-density lipoproteins (HDL) and their major apolipoprotein (apo)A-I pass the endothelial cell barrier by mechanisms involving the scavenger receptor (SR)-BI, the ATP binding cassette transporters (ATP) A1 and G1, the endothelial lipase (EL) and the ectopic β-ATPase. However little is known on how inflammation influences this trans-endothelium transport.

Approach and results: Upon stimulation with interleukin (IL)-1β or tumor necrosis factor (TNF)α primary endothelial cells demonstrated decreased binding and transport of apoA-I. In addition β-ATPase cell surface expression was reduced modestly reduced TNFα but markedly decreased by IL-1β. The ABCA1 expression was up regulated by either both cytokines. In addition, IL-1β increased the expression of ABCG1 and EL and decreased the expression of SR-BI whereas HDL binding or transport were not changed. TNFα induced the expression of EL and ABCG1 as well as the binding of HDL to the endothelial cells. Nevertheless the specific transport through endothelial cells was decreased. This discrepancy between binding and transport of HDL after TNFα stimulation was not explained by enhanced cellular HDL degradation but by reduced internalization.

Conclusion: TNFα and IL-1β reduce the translocation of apoA-I and HDL through the endothelium, the first step in reverse cholesterol transport pathway by regulating different steps in the cellular interaction of HDL and apoA-I with cells.

Introduction

Atherosclerosis is the major cause of morbidity and mortality worldwide. It is characterized by lipid retention within the arterial wall forming atherosclerotic plaques. Moreover a general inflammatory state is generally associated within these plaques. Indeed many inflammatory markers are expressed in the plaque, among them the pro-inflammatory cytokines: interleukin 1β (IL-1β) and the tumor necrosis factor α (TNFα) [1,2]. The secretion of these cytokines is caused by the presence in the plaque of lymphocyte T cells and cholesterol-loaded macrophages, the macrophage foam cells [3]. Many studies revealed the inverse correlation of high density lipoproteins (HDL) concentration in the plasma and the risk of atherosclerosis [4]. The best investigated, potentially athero-protective role of HDL and its major apolipoprotein (apo) A-I is their ability to remove cholesterol from macrophage foam cells in the arterial wall to carry it to the liver for excretion into the bile [5,6]. An early step in the reverse cholesterol transport is the transfer of cholesterol from the macrophages to the lipid free apoA-I and HDL. Importantly the loading of cholesterol is not taking place in the plasma but in the arterial intima [6]. Consequently HDL has to cross the endothelium to get access to the cholesterol loaded cells. We previously demonstrated that endothelial cells bind, internalize and transcytose HDL as well as apoA-I in a saturable and temperature dependent manner. Using siRNA and pharmacological interferences we demonstrated that endothelial cells bind and transport HDL by distinct specific mechanisms involving the scavenger receptor (SR)-BI, the ATP binding cassette transporter (ABC) G1 [7] and endothelial lipase (EL) whereas lipid free apoA-I interact with ABCA1 and the ectopic β-ATPase [8,9].

Since atherosclerosis is an inflammatory state, we here investigated whether TNFα and IL-1β modulate the transport of lipid free apoA-I and HDL through endothelial cells.

Material and methods

Endothelial cells cultivation and treatment

Bovine aortic endothelial cells were cultivated as previously described [8,10]. Endothelial cells were incubated with IL-1β or TNFα (Preprotech, USA) at a final concentration of 10 ng/ml in DMEM (Sigma Aldrich) containing 0.2% BSA prior the assay for 24 or 48 hours.

Endothelial binding, cell association and transport of HDL

The methods for the isolation and radiolabeling of apoA-I and HDL as well as the quantification of endothelial binding, association, and transport of HDL have been described previously [7,8,10]. In brief, endothelial cells were incubated with 5 μg/ml of ^{125}I-apoA-I or 10 μg/ml of ^{125}I-HDL without (total) or with a 40 fold excess of non-labeled HDL (non-specific) for 1 hour at 4 °C for cell binding and at 37 °C for cell association or for transport studies in transwell systems. Specific binding/association/transport rates were calculated by subtracting the respective unspecific rates obtained in the presence of an excess unlabeled apoA-I or HDL as the competitors from the respective total rates observed in the absence of any competitor. All experiments were done in at least three triplicate experiments.

HDL degradation

The assay was performed as previously described [10]. In brief, the cells were incubated with 10 μg/ml of ^{125}I-HDL in the absence or presence of 40-fold excess of non-labeled HDL. After incubation for 4 hours at 37 °C the amount of ^{125}I-HDL degradation products released into the medium was measured. The proteins in the incubation medium were precipitated with trichloroacetic acid (TCA). To distinguish the cellular degradation products from the free iodine in solution after TCA precipitation, the supernatant was oxidized by hydrogen peroxide and the free iodine extracted with trichloroethane. The radioactivity in the aqueous phase containing the cellular degradation products was measured using a Perkin Elmer γ-counter.

HDL Internalization

The assay was performed as described above for binding and cell association studies. After 1 hour incubation ^{125}I-HDL in DMEM containing hepes with 0.2 % BSA, the cells

were washed twice with ice-cold PBS containing 0.1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ and incubated on ice for 20 minutes. The cell surface proteins were biotinylated at 4 °C with sulfo-NSH-biotin (Pierce) (250 g/mL) in PBS (Ca^{2+} and Mg^{2+}). After 1 hour the reaction was terminated by 10 minutes incubation with DMEM. After two additional PBS washes the cells were scrapped and solubilized in RIPA buffer (10 mmol/L Tris pH 7.4, 150 mmol/L NaCl. 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, complete EDTA (Roche)). The supernatant was isolated after a 10 min spin at 10000 x g and a portion was used for total protein and radioactivity quantifications and the remained was incubated with streptavidin- conjugated sepharose beads (Amersham Biosciences) at 4°C over-night. The streptavidin beads binding the cell surface proteins were precipitated by centrifugation and the supernatants containing the cytoplasmic proteins were further analyzed. The radioactivity of the supernatant was measured using Perkin Elmer γ-counter. The amount of cell surface bound HDL was calculated by subtracting the internalized to the total cells association.

Endothelial cell monolayer permeability

The tightness of the endothelial cell monolayer was assessed as ^{3}H-Inulin filtration as previously described [10]. In brief, endothelial cells were cultivated in transwells system for two days before stimulation. After 48 hours stimulation with 10 ng/ml of IL-1β or TNFα, the permeability was measured. ^{3}H-Inulin was added to the upper chamber and the medium of the lower and the upper chamber were collected after one hour at 37° C and measured with a β-counter. Permeability was calculated as described by Youdim et al [11].

Western Blotting

Total proteins were analyzed after lysing endothelial cells in RIPA buffer. Cell surface proteins were analyzed as previously described [8,9]. In brief, cell monolayers were biotinylated with EZ-linked Sulfo-NHS-SS-biotin (Pierce) (250 μg/mL) in PBS containing 0.1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ at 4 °C for one hour. The reaction was terminated by 10 minutes incubation in DMEM at 4 °C and cells were lysed in RIPA buffer. The lysates were incubated with streptavidin- conjugated sepharose beads (Amersham Biosciences) at 4°C overnight. The beads were washed three times with the lysis buffer and the bound proteins were separated from the beads by incubation with 5

mM DTT at room temperature for one hour. Protein concentrations were determined and equal amounts of proteins were separated on a SDS-PAGE and trans-blotted onto a PDVF membrane (GE Healthcare). The protein expression was analyzed with specific antibodies against ABCA1 (anti ABCA1 AB.H10 ABCAM), β-ATPase (anti β-ATPase MS503 MitoScience, Eugene, OR), ABCG1 (anti ABCG1 E20 Santa Cruz), SR-BI (anti SR-BI E4 Novus or anti SR-BI EP1556Y ABCAM) and EL (anti EL NB 400-118 Novus) and compared to the expression of actin (anti-actin AC-15, Sigma). Intensity of the blot was quantified using Image J.

Immunofluorescence

Endothelial cells were seeded on cover-slides two days prior the assay. Then cells in DMEM with 0.2 % BSA were incubated with 50 nM of LysoTracker® probe (Invitrogen) followed by 25 μg/ml of HDL previously labeled with atto-488 (Sigma Aldrich) following the manufacturer's instructions. After one hour incubation, cells were washed three times in PBS containing 0.1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ and fixed with 3.75 % paraformaldehyde (PFA) at room temperature for 30 minutes. The fixation was stopped by washing with 0.5 mM Tris-HCl pH 7.4 solution for 10 minutes. After washing with PBS twice, cells were mounted in embedding solution containing DAPI to visualize the nuclei (Invitrogen). The cells were visually inspected using an axiovert 200M microscope (Zeiss).

Statistical analysis

The data for all experiments were analyzed using Graph Pad Prism 5 software program. Comparisons between groups were performed using t-test or ANOVA. Experiments were routinely performed in triplicate. The data have been obtained from at least three individual experiments and are graphically represent as means ± standard deviation (SD). n.s. not significant; * $P<0.05$; ** $P<0.001$; *** $P<0.0001$

Results

TNFα and IL-1β reduce apoA-I binding and transport

To investigate whether the interaction of apoA-I with endothelial cells is modulated by inflammatory cytokines we stimulated aortic endothelial cells with TNFα and IL-1β. The interactions of iodinated apoA-I with the endothelial cells were characterized as binding at 4 °C, cell association and transport at 37 °C. After 48 hours stimulation with the cytokines the specific binding was compared to not stimulated cells. It was significantly reduced to 73 ± 18% and to 43 ± 20% in TNFα and IL-1β stimulated cells respectively (Fig. 1A). TNFα and IL-1β also reduced the specific cell association of apoA-I to 77 ± 11% and to 74 ± 15% respectively (Fig. 1B). Interestingly apoA-I transport was also significantly reduced to 48 ± 32% after IL-1β stimulation. In contrast the specific transport of apoA-I after TNFα treatment was not changed significantly (84 ± 40%) (Fig. 1C). Previous studies reported that inflammatory cytokines induced endothelial cell barrier permeability. Therefore the integrity of the endothelial cells monolayer was assessed by analyzing the permeability with ^{3}H-Inulin after TNFα and IL-1β stimulation. The integrity of the endothelial monolayer was not significantly changed after IL-1β stimulation compared to not stimulated cells, 1.683 E^{-5} ± 9.10 E^{-7} and 1.782 E^{-5} ± 7.318 E^{-7} respectively. By contrast, TNFα stimulation increased the permeability of the endothelial cell monolayer by ≈ 20% respectively 2.111 E^{-5} ± 1.325 E^{-6} (Fig. 2).

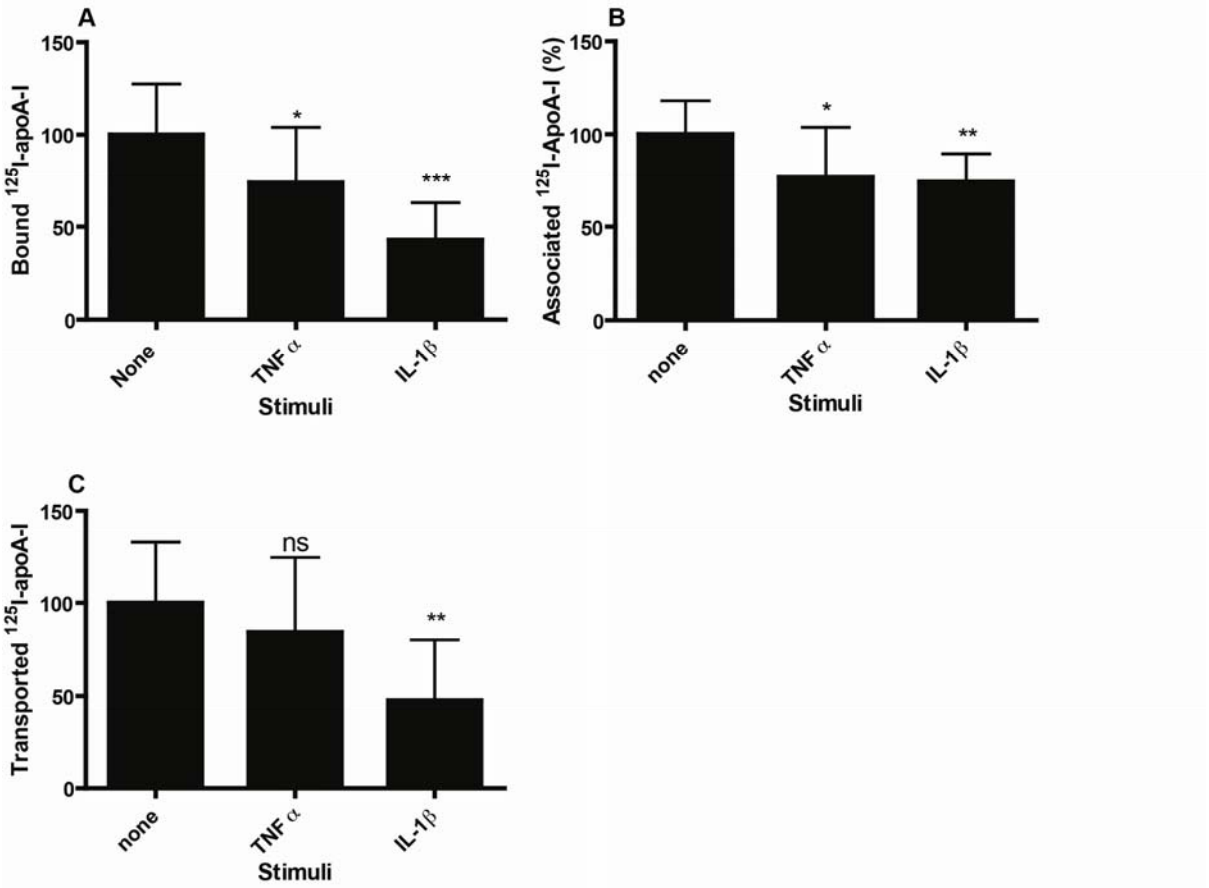

Figure 1. Influence of TNFα and IL-1β on specific endothelial binding at 4°C (A), cell association at 37°C (B), and transport of apoA-I (C). *ECs were stimulated with TNFα or IL-1β (10 ng/ml) for 48 hours prior the assays. Binding (A) was measured by incubating the cells with 5 μg/ml ^{125}I-apoA-I $\pm$ 40 folds excess of un-labeled apoA-I at 4 °C for 1 hour. To analyze the association (B) and transport (C) ECs were cultivated for 24 hours on insert before TNFα or IL-1β stimulation then ^{125}I-apoA-I $\pm$ 40 folds excess of un-labeled apoA-I was added to the apical compartment. After one hour at 37 °C cells were lysed and specific activity was counted to obtain cell association (B). Medium of the basal compartment was harvested and specific activity was monitored to obtain the transport (C). The results represent specific binding, association and transport which were calculated as the differences of radioactivity recorded in the absence of competitor minus radioactivity recorded in the presence of competitors. They represented as means $\pm$ SD of at least 3 individual experiments made of triplicate.*

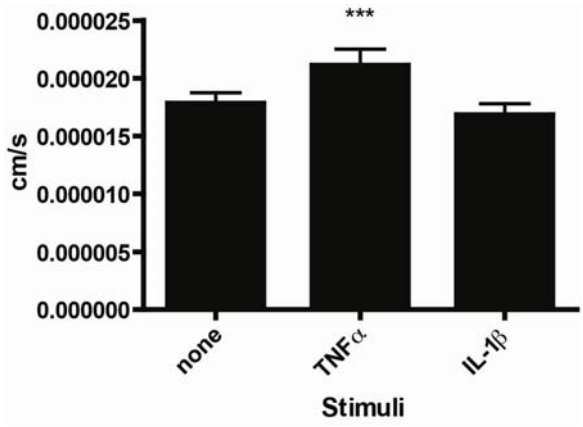

Figure 2. Influence of TNFα and IL-1β on endothelial permeability. *ECs were stimulated with TNFα or IL-1β (10 ng/ml) for 48 hours prior the assays. The permeability of the cell monolayer to inulin was determined. The results are represented as mean ± SD of at least 3 individual experiments made of triplicate*

TNFα and IL-1β do not reduce ABCA1 but reduce cell surface expression of β-ATPase.

Previously, we demonstrated that ABCA1 and the ectopic β-ATPase modulate the binding of apoA-I to the endothelial cells. Therefore we analyzed by Western blotting if IL-1β and TNFα influence the expression of ABCA1 and β-ATPase in the cells. After 48h the total protein expression as well as the cell surface expression level of ABCA1 was increased after both TNFα and IL-1β stimulation (Fig. 3). In the total cell lysate no change in the expression of the β-ATPase was observed. However on the cell surface the expression level was moderately reduced after TNFα stimulation but markedly reduced after IL-1β stimulation compared to not stimulated cells.

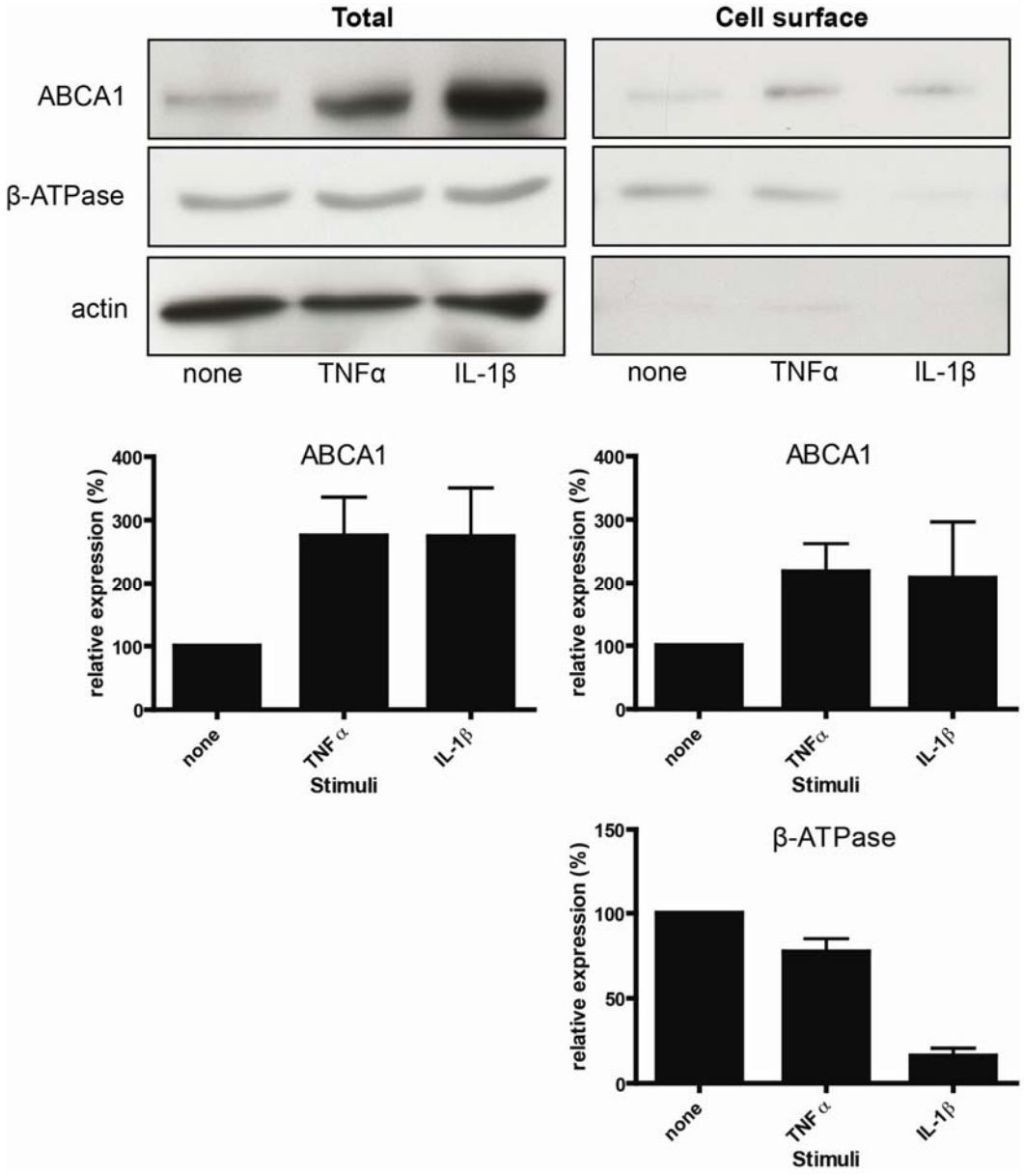

***Figure 3. Protein expression of ABCA1 and β-ATPase after stimulation with TNFα or IL-1β (10 ng/ml; 48 h**). The protein levels of ABCA1 and β-ATPase were evaluated by western blotting. Cell surface proteins were differentiated from the total proteins by cell surface biotinylation. Representative blots and quantifications of band intensities of at least 3 experiments are presented. The results are represented as mean ± SD of at least 3 individual experiments*

TNFα not IL-1β modulates HDL binding, cell association and transport through the endothelial cells.

To investigate whether inflammatory cytokines influence HDL binding, association and transport, endothelial cells were stimulated with TNFα or IL-1β for 24h. The stimulation of the cells with IL-1β did not change the binding, association and transport of HDL significantly (Fig. 4 A-C). Interestingly, TNF-α stimulation increased binding and cell association of HDL to endothelial cells by 166 ± 62% ($p<0.01$) and 134 ± 29% (not significant), respectively compared to not stimulated cells (Fig 4 A-B). In contrast HDL transport was strongly reduced to 44 ± 24% (Fig. 4 C).

The discrepancy between increased HDL binding and decreased transport in endothelial cells after TNFα stimulation suggested that TNFα either induces intracellular HDL degradation or reduces HDL up-take. To analyze the first hypothesis, the degradation of ^{125}I-HDL was measured after TNFα stimulation. However no significant enhancement of HDL degradation could be observed in cells stimulated with TNF-α as compared to not-stimulated cells 10.26 ± 0.38 and 10.33 ± 1.15 ng/mg protein respectively (Fig. 5 A). This finding was further confirmed by incubating endothelial cells with fluorescently labeled HDL and Lysotracker® probe, which revealed no co-localizing of HDL with the lysosomes in either TNFα stimulated or in none stimulated cells (Fig. 5 B). The incubation of the cells with fluorescently labeled HDL demonstrated a similar cellular distribution pattern of HDL in the TNF-α stimulated and not stimulated cells but the signal appeared to be decreased after TNFα stimulation. Therefore the cellular distribution of ^{125}I-HDL was analyzed by cell surface biotinylation. Interestingly, HDL cell-association (cell surface bound and internalized HDL) was not changed after TNFα stimulation (112 ± 23%) compared to not stimulated cells (100%). But the internalized HDL was clearly reduced compared to not stimulated cells 50 ± 5% and 66 ± 12% respectively. In agreement with the previous binding studies, cell surface bound HDL was increased 1.8 fold after TNFα stimulation (62 ± 23%) compared to none stimulated cells (34 ± 16%) (Fig. 5 C)

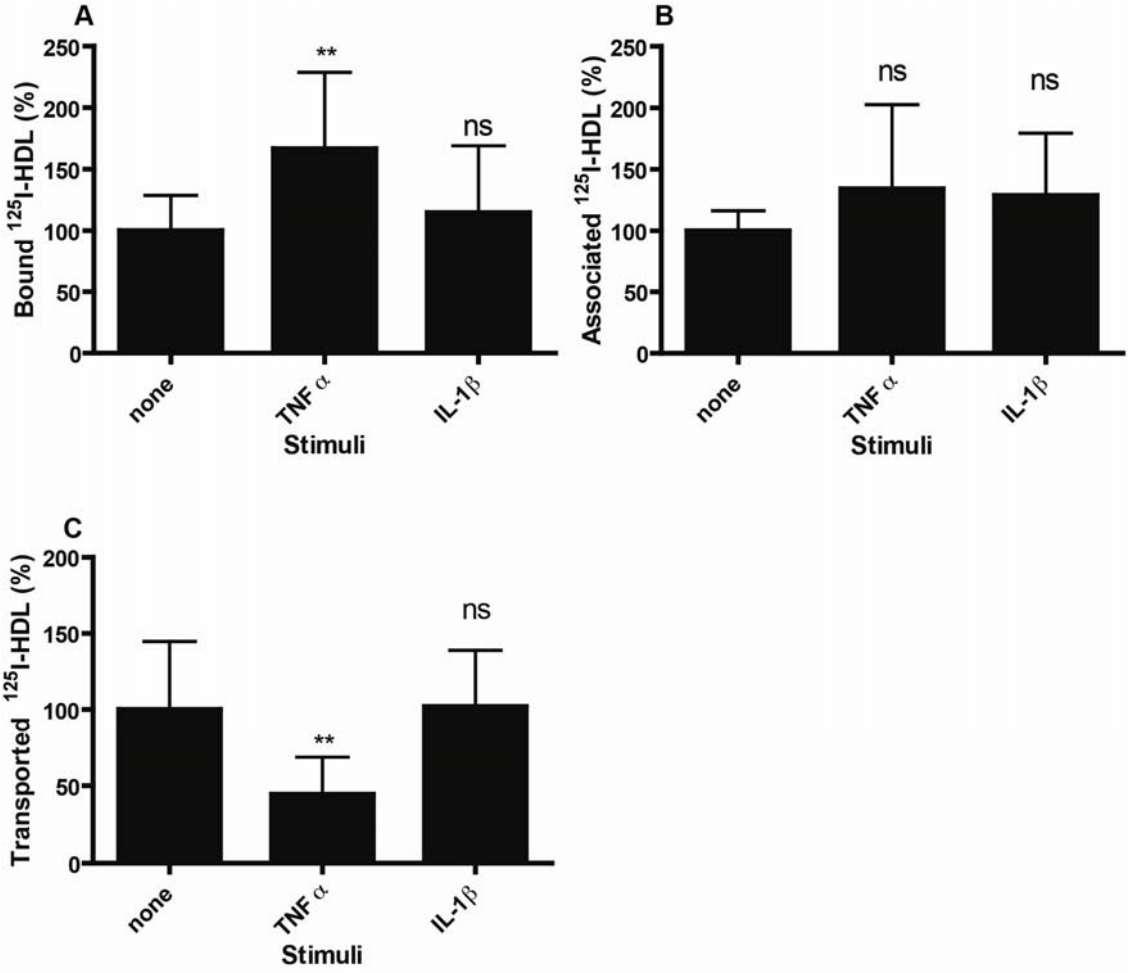

Figure 4. Influence of TNFα and IL-1β on specific endothelial binding at 4°C (A), cell association at 37°C (B), and transport of HDL (C). *ECs were stimulated with TNFα or IL-1β (10 ng/ml) for 24 hours prior the assays. Binding (A) was measured by incubating the cells with 10 μg/ml ^{125}I-HDL $\pm$ 40 folds excess of un-labeled HDL at 4 °C for 1 hour. To analyses the association (B) and transport (C) ECs were cultivated for 24 hours on insert before TNFα or IL-1β stimulation then ^{125}I-HDL $\pm$ 40 folds excess of un-labeled apoA-I was added to the apical compartment. After one hour at 37 °C cells were lysed and specific activity was counted to obtain cell association (B). Medium of the basal compartment was harvested and specific activity was monitored to obtain the transport (C). The results represent specific binding, association and transport which were calculated as the differences of radioactivity recorded in the absence of competitor minus radioactivity recorded in the presence of competitors. They represented as means $\pm$ SD of at least 3 individual experiments made in triplicate.*

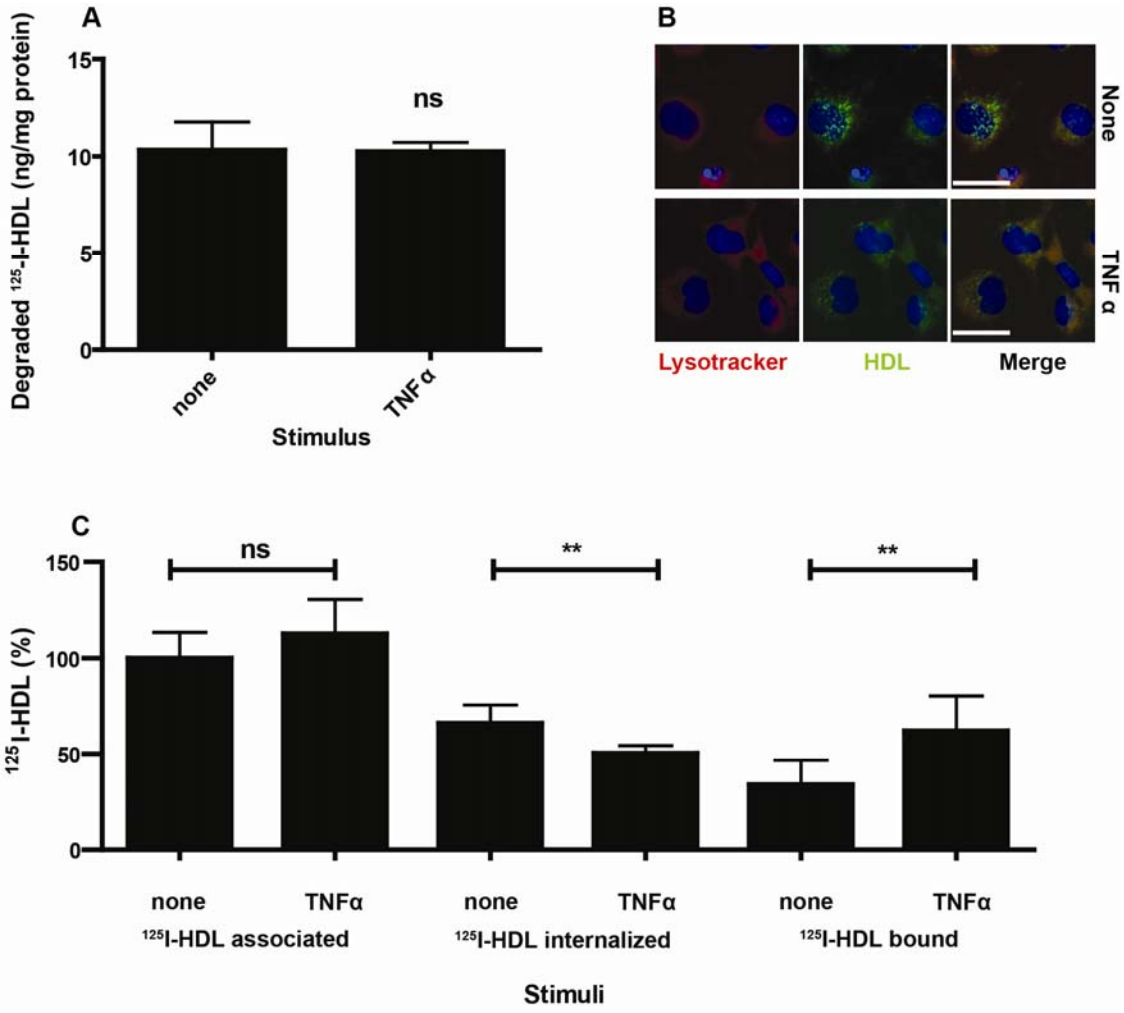

Figure 5. Influence of TNFα on HDL degradation (A-B) and HDL uptake (C). *ECs were stimulated with TNFα or IL-1β (10 ng/ml) for 24 hours prior the assays. Degradation (A) was measured by incubating the cells with 10 μg/ml ^{125}I-HDL at 37 °C for 4 hours. Intracellular localization of HDL (B) was monitored by incubating cell with 25 μg/ml of fluorescently labeled HDL (green) and 50 nM of Lysotracker® (red) for one hour at 37 °C (bars = 50μm). Uptake (C) was measured by incubating the cells with 10 μg/ml ^{125}I-HDL at 37 °C. After one hour cells were washed and incubated with biotin as described in the method. The results are represented as mean ± SD of at least 3 individual experiments made of triplicate.*

TNFα and IL-1β modulate the expression of ABCG1, SR-BI and EL

We next investigated the effect of inflammatory cytokines on the expression of ABCG1, SR-BI and EL in endothelial cells. The protein expression analysis by western blotting demonstrated that both TNFα and IL-1β induce the expression of both ABCG1 and EL. The expression of SR-BI was reduced after IL-1β stimulation.

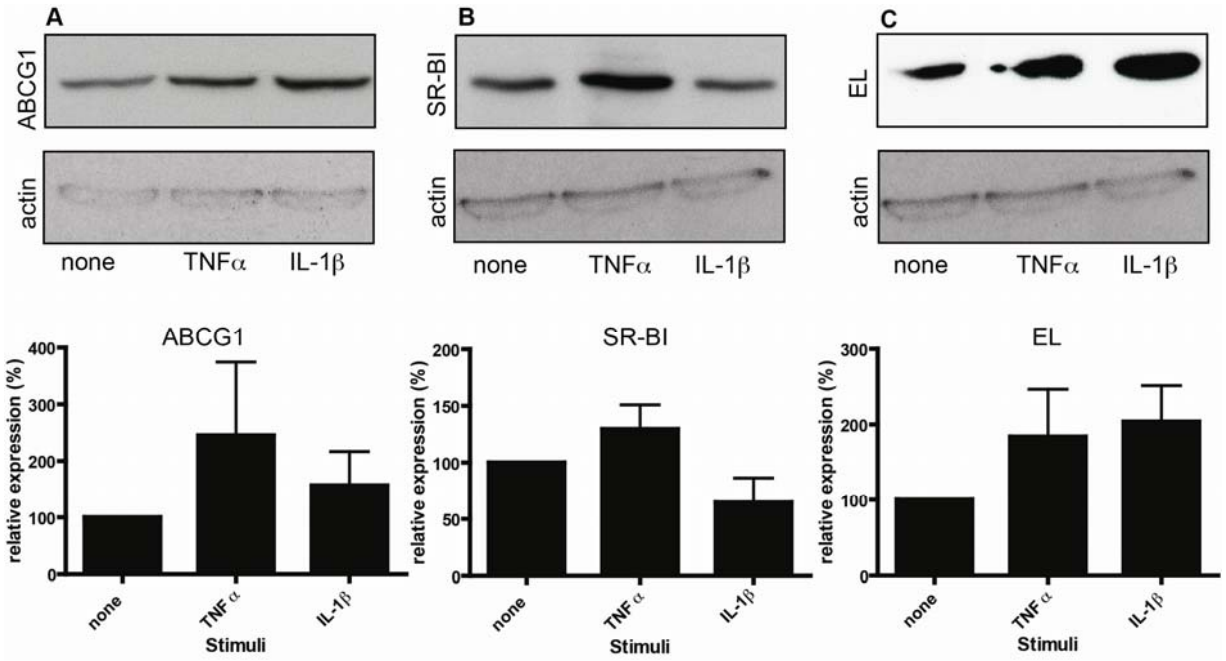

Figure 6. Protein expression of ABCG1 (A), SR-BI (B) and EL (D) after stimulation with TNFα or IL-1β ((10 ng/ml; 48 h). *The protein levels of ABCG1, SR-BI and EL were evaluated by western blotting. Representative blots and quantifications of band intensities of at least 3 individual experiments are presented. The results are represented as mean ± SD of at least 3 individual experiments*

Discussion

Several lines of evidence suggest the essential role of TNFα and IL-1β in atherosclerosis development. Indeed mouse models deficient of either TNFα or IL-1β develop less atherosclerotic lesions [12–14] whereas mice exposed to an excess of inflammatory cytokines develop more atherosclerosis [15]. Human studies also supported the pro-atherogenic role TNFα and IL-1β. Indeed circulating levels of either cytokine were shown to increase the risk of atherosclerotic cardiovascular events [16,17]. Finally TNFα and IL-1β are present in atherosclerotic plaques [1,2].

The effect of inflammatory cytokines on the physiology of endothelial cells has been extensively studied in the last decades. We here investigated for the first time the effects

of TNFα and IL-1β on the transport of apoA-I and HDL through endothelial cells. TNFα and IL-1β reduced both binding and cell association of lipid-free apoA-I in endothelial cells. In addition apoA-I transport was reduced after IL-1β stimulation but not altered after TNFα incubation. In agreement with previous reports [18–21] TNFα increased the endothelium permeability by 20%. Despite this increase leakage, we did not observe any increase in the transport of apoA-I and HDL transport was rather decreased. This underscores the robustness of our method to assess specific transport of endothelial cells.

Interestingly stimulation with IL-1β did not change the permeability and the transport capacity was reduced as expected upon the reduction in the apoA-I binding. We previously reported that apoA-I binds to both ABCA1 and the ectopic β-ATPase [8,9]. apoA-I is lipidated by ABCA1 whereas it activates β-ATPase to generate ADP which is in turn stimulates the $P2Y_{12}$ receptor to signal to an as yet unknown target activating the uptake of lipidated apoA-I or HDL [9,22]. In the present study we demonstrated that TNFα and IL-1β induce ABCA1 expression but reduce β-ATPase expression on the plasma membrane. Only the reduced expression of the ectopic β-ATPase is in agreement with the reduced apoA-I binding to endothelial cells observed after IL-1β stimulation. The reduced cell association and transport after stimulation with IL-1β further supports the essential role of the apoA-I/β-ATPase→$P2Y_{12}$ receptor signaling for uptake of lipidated apoA-I or HDL. However further experiments are needed to proof, that IL-1β interferes with HDL transport via this cascade.

We previously reported that upon stimulation with IL-6, HDL binding to endothelial cells was enhanced dependently of the up-regulation of EL which is in line with reports demonstrating induced EL expression in inflammation [23–25]. In the present study TNFα and IL-1β also induced EL expression. However TNFα but not IL-1β enhanced HDL binding to the endothelial cells. This discrepancy could be explained by the reduction of SR-BI expression after IL-1β stimulation, which may counteract the effect of EL up-regulation. Also TNFα induced HDL binding is explained by increased EL up-regulation. However, despite increased binding HDL transport was reduced by TNFα due to a reduction of HDL internalization. Likewise TNFα was demonstrated to reduce insulin

uptake in aortic endothelial cells [26]. The discordance between increased binding of HDL potentially due to enhancement of EL abundance on the cell surface and reduced internalization points to the presence of a rate limiting step in trans-endothelial HDL transport which is not mediated by the as yet known HDL binding proteins SR-BI, EL and ABCG1.

In conclusion our study demonstrated that the inflammatory cytokines TNFα and IL-1β reduced apoA-I and HDL trans-endothelium transport. Further experiments will be essential to analyze signaling pathways involve in this process.

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Chapter

4

A three-dimensional engineered artery model for atherosclerosis research

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Short title: *Artery engineering to study atherosclerosis in vitro.*

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*** Manuscript in preparation**

Contribution JR:
Participated to the experimental design, performed most of the analyses and wrote the manuscript

Abstract

The pathogenesis of atherosclerosis involves dysfunctions of vascular endothelial cells and smooth muscle cells as well as blood borne inflammatory cells such as monocyte-derived macrophages. In vitro experiments towards a better understanding of these dysfunctions are typically performed in two-dimensional cell culture systems. However, these models lack both the three-dimensional structure and the physiological pulsatile flow conditions of native arteries. We here describe the development and initial characterization of a tissue engineered artery equivalent, which is composed of human primary endothelial and smooth muscle cells and is exposed to flow in vitro. Histological analyses showed formation of a dense tissue composed of a tight monolayer of endothelial cells supported by a basement membrane and multiple smooth muscle cell layers. Both low (LDL) and high density lipoproteins (HDL) perfused through the artery equivalent were recovered both within endothelial cells and in the sub-endothelial intima. After activation of the endothelium with either tumor necrosis factor alpha (TNFα) or LDL, monocytes circulated through the model were found to adhere to the activated endothelium and to transmigrate into the intima.

In conclusion, the described tissue engineered human artery equivalent model represents a significant step towards a relevant in vitro platform for the systematic assessment of pathogenic processes in atherosclerosis.

Introduction

Atherosclerosis is responsible for a substantial global disease load and remains a major cause of morbidity and mortality worldwide. One of the first events in the pathogenesis of atherosclerosis is the insudation of cholesterol-rich low-density lipoprotein (LDL) into the innermost intimal layer of the arterial wall where they are bound by proteoglycans and eventually modified [1]. In parallel and further enhanced by the retained lipoproteins, the activated endothelium binds immune cells such as monocytes, which subsequently transmigrate the endothelium. Within the arterial intima the monocytes differentiate into macrophages which upon receptor mediated uptake of LDL are transformed into foam cells. They form the early "fatty streak" lesion [2]. The inflammatory reaction of macrophage foam cells but also other immigrating immune cells induce the progression to more complicated lesions and ultimately vulnerable plaques which upon rupture or erosion elicit the acute complications of atherosclerosis such as acute coronary events. High density lipoproteins (HDL) counteract several of these pro-atherogenic activities by various mechanisms. The classical potentially anti-atherogenic property is related to its ability to remove cholesterol from the macrophage foam cells within the arterial wall and mediate the transport to the liver for excretion into the bile [3]. To do this HDL must also cross the endothelial barrier in order to get access to the cholesterol-loaded macrophages [3].

In addition to various in vivo models, notably genetically modified mice, [4] cell culture experiments have been valuable tools to unravel the above summarized pathomechanisms of atherosclerosis. However - so far - most of the in vitro experiments are performed using single types of cells grown on regular static plastic dishes. Importantly, these studies are associated with significant limitations, as they do not account for the complexity of the native artery environment with all the cell-cell and/or cell-matrix interactions. To address these limitations, several laboratories developed co-culture models of endothelial cells and smooth muscle cells [5–7]. However, also these studies were limited by the unphysiological attachment of cells to plastic dishes or trans-well membranes and co-culture times which were too short for the development of typical vascular cell-extracellular matrix interactions. In the attempt to address these limitations, Dorweiler et al. developed a long-term co-culture setup on fibrin gels [8]. In this

model they demonstrated for the first time the feasibility to analyses in vitro the accumulation of LDL and immunocytes in a sub-endothelial matrix [8,9]. However, also this static and non-dynamic co-culture system is limited by the lack of both the circular structure of arteries and the physiological vascular hemodynamic situation characterized by flow and shear-stress.

In recent years, several laboratories fabricated tissue engineered artery equivalents under pulsatile, native-like flow conditions for the therapeutic and regenerative repair of congenital and acquired malformations [10–13]. These bioengineered autologous cell-based constructs were also successfully implanted into large animal models and revealed native-like behavior and development up to 240 weeks in vivo [14]. In the present study we used human vascular cells to engineer a 3D artery model mimicking the structural as well as functional characteristics of a native artery. This native-analogous bioengineered artery model was used to study initial events in atherosclerosis, namely the accumulation of LDL and HDL in the intima as well as the binding and transmigration of monocytes under dynamic pulsatile flow conditions.

Methods

Isolation of Umbilical cord cells

Mature vascular cells were isolated from human umbilical cord vessels. All experiments were performed under cantonal ethical permission and informed consent was obtained from all patients [KEK-2009-95]. Human umbilical vein endothelial cells (HUVECs) and human umbilical cord derived myofibroblasts (UCMFBs) were isolated and characterized as previously described [10,14]. In brief, HUVECs were isolated using the collagenase instillation technique. For this, the umbilical cord vessels were incubated in collagenase (2 mg/ml; Collagenase A; Roche Diagnostics Gmbh.) dissolved in serum-free medium (EBM™; LONZA Inc., Switzerland). After 20 min of incubation, the cell suspension was centrifuged and isolated cells were expanded in EGM™-2 medium (supplemented with VEGF, hrIGF-1, hEGF, gentamycin, amphotericin-B, hydrocortisone, ascorbic acid, heparin, and 2% FBS; LONZA Inc., Switzerland). For the isolation of UCMFBs the

remaining vessels were minced into small pieces (~2-3 mm) and incubated without medium under the sterile laminar flow for 25-30 min to ensure physical attachment of the minced pieces. Subsequently, advanced DMEM medium (Invitrogen Corp., USA; supplemented with 10% FBS; 0.05% Penicillin/Streptomycin, 0.02 % Fungizone and 1% L-glutamine) was carefully added to the minced vessel pieces and the adherent myofibroblastic cells were expanded up to passage 6.

In vitro fabrication of tissue engineered vascular grafts

Biodegradable tubular scaffold matrices (length 4 cm and inner diameter 0.6 cm) were fabricated as described previously[10]. Briefly, non-woven polyglycolic-acid meshes (PGA; Cellon, Luxembourg) were dip-coated with poly-4-hydroxybutyrate (P4HB; TEPHA Inc., USA) by dipping it into a 1.75 % (w/w) solution of P4HB/tetrahydrofuran solution (Sigma Aldrich). Vascular shape was obtained by heat application and a welding technique before external coating with 10 % P4HB/tetrahydrofuran solution. After sterilization with 70 % ethanol (30 min) followed by 3 washes with PBS or by ethylene oxide exposition, PGA-P4HB composite scaffolds were pre-incubated in DMEM medium over night before cells seeding. UCMFBs (3-4x10^6 cells/cm^2) were seeded in the inner surface of the vascular scaffold using fibrin (Sigma Aldrich) as a cell carrier [15]. After a short static incubation period (3 days), vascular constructs were exposed to dynamic conditioning in a flow bioreactor system for 14 days. The flow of nutrient medium (DMEM with 10% FBS; 0.05% Penicillin/Streptomycin, 0.02 % Fungizone, 1 % L-glutamine and 1.5 mM L-ascorbic acid) was directed through their inner lumen of the bioreactor circulation loop, exposing the seeded constructs to an increasing flow other time (from 5 to 10 mL/min). Vascular grafts were then endotheliazed with HUVECs (1.5x10^6 cells/cm^2) and cultivated first in static condition for 5 days in EGM™-2 medium with supplements as described above. After the static phase, vascular grafts were placed back in the bioreactor for 14 additional days with increasing medium flow (from 3 to 10 mL/min).

Isolation and labeling of LDL and HDL

Human LDL (1.006 < d < 1.063 kg/L) and HDL (1.063 < d < 1.21 kg/L) were isolated from fresh normolipidemic plasmas of blood donor by sequential gradient ultracentrifugation [16]. The purity of the lipoprotein preparation was verified by SDS-PAGE in order to assure no cross or albumin contamination. Freshly isolated LDL or

HDL was fluorescently labeled with atto-633 (Invitrogen) following manufacturer instructions. Fluorescent LDL (20 μg/ml in EGM™-2 medium supplemented as described previously) was injected in the circulatory loop of the bioreactor in the absence or presence of a 50 folds excess (1 mg/ml) of non-labeled LDL. After 2.5 or 24 hours vascular grafts were removed and processed directly using confocal microscopy (CLSM SP5, Leica) or frozen in embedding matrix (O.C.T., Biosystem) for further analyses. Fluorescently labeled HDL (25 μg/ml in EGM™2 medium supplemented as described previously) was injected into the circulatory loop of the bioreactor system or injected into the medium adherently cultured cells. After 4 or 24 hours tissue engineered vascular grafts were removed and further processed as described for LDL.

Isolation and labeling of monocytes

Monocytes were isolated from healthy donors by centrifugation on a continuous density gradient (Percoll™; Amersham bioscience, GE health care) as described previously [17]. Freshly isolated monocytes were fluorescently labeled with 10 μM of SNARF-1 carboxyl acid (Invitrogen) following the manufacturer's instructions. $1x10^6$ monocytes per mL were injected into the circulation loop of the bioreactor in EGM™-2 medium supplemented as described above but with 2 % of FBS.

Histology and immunohistochemistry of tissue engineered arteries

For histological characterization bioengineered grafts were fixed in 4%-paraformaldehyde (PFA), dehydrated through a series of graded ethanol, embedded in paraffin and sectioned at 7 μm thickness. The sections were deparaffinized, rehydrated through a graded ethanol series. The tissue sections were stained using Haematoxylin & Eosin, Haematoxylin-Sudan and Masson-trichrome. Immunohistochemistry analyses were performed with antibodies specific for CD31 (clone JC/70A, DakoCytomation, Glostrup, Denmark), α-smooth muscle actin (clone 1A4, Sigma) and collagen IV (Quartett, Germany) using the Vantana Benchmark automated staining system (Ventana Medicals Systems, Tucso,AZ) as previously described [10].

Biochemical extracellular matrix analysis

Representative tissue samples were lyophilized and analyzed by biochemical assays for total DNA content as an indicator for cell number, hydroxyproline (Hyp) content as an

indicator for collagen, as well as glycosaminoglycans (GAG) content. For measuring the DNA amount, the Hoechst dye method [18] was used with a standard curve prepared from calf thymus DNA (Sigma Chemical Co., USA). The GAG content was determined using a modified version of the protocol described by *Farndale* et al. [19] and a standard curve prepared from chondroitin sulphate (Sigma Chemical Co., USA). HYP was determined using a modified version of the protocol provided by *Huszar* et al. [20]. Native control tissues from human aorta were included and all values are presented relative to these native values (% of native).

Confocal microscopy

Bioengineered grafts were first washed twice in PBS and then fixed in 3.75 % of PFA for 30 minutes at room temperature. Following a second series of PBS washing phases (3x), grafts were mounted in 0.1 M Tris-HCL, pH 9.5, and glycerol (3:7) containing 50 mg/ml of n-propyl gallats as anti-fading reagent [21] and 1 ng/ml of DAPI to visualize the nuclei. Analyses of the engineered graft samples were performed on an inverted SP5 confocal microscopy (Leica).

Preparation of the cryopreserve sections

Bioengineered grafts were first washed twice in PBS and then cryopreserved in embedding matrix (O.C.T.). The grafts were further processed on cryotome generating cuts with 12 μm thickness. The cuts were stored at -80 °C until further processing. Cuts were rehydrated in PBS for 3 x 10 minutes before fixing in 3.75% of PFA for 20 minutes at room temperature. After three PBS washes cuts were blocked for 30 minutes in 5% donkey serum in PBS. For immunofluorescence staining, the cuts were incubated overnight at 4 °C with specific antibodies against ZO1 (33-9100, Invitrogen), ABCG1 (HG5, Santa Cruz), SR-BI (NB 400-101 E4, Novus) or EL (NB 400-111, Novus). After three additional PBS washes cuts were incubated for 45 minutes at room temperature with secondary antibodies (Dako) followed by three additional washes with PBS and finally mounted in antifade solution containing DAPI (Invitrogen). The expression of ABCG1, SR-BI and EL were compared to a native radial artery frozen in embedding matrix (O.C.T.) and cut as describe above. Analyses of the cuts were performed on an inverted axio 200M microscope (Zeiss).

Endothelium integrity

To analyses the endothelial integrity Evan's blue (Sigma Aldrich) was injected at a final concentration of 0.5% in the circulation loop of the bioreactor for 10 minutes followed by PBS washing phase for 10 minutes. Grafts were cut open and analyzed macroscopically with photo documentation.

Statistical analysis

Statistical comparisons between different groups were performed using a paired t-test. The data has been obtained from at least 2 different experiments and were graphically represented as mean ± standard deviation of the mean (SEM). P-values of < 0.05 were considered statistically significant. All statistical analyses were performed using Graph Pad Prism-5 software. n.s. not significant; * $P<0.05$; ** $P<0.001$; *** $P<0.0001$

Results

Structure and characteristics of engineering arteries

For the assessment on the structural level, the engineered artery equivalents were analyzed macroscopically and microscopically. Macroscopically the tube-like scaffolds had dimensions of 4 cm length and about 0.6 cm diameter before seeding. After the in vitro culture for about 5 weeks the scaffold was densely covered by cells and demonstrated a patent lumen (Fig. 1). Some of the grafts (15 %) showed a central decrease of the luminal diameter. These grafts were excluded from any further analyses.

Microscopy analyses with Haematoxylin-Sudan and Haematoxylin-Eosin staining demonstrated the formation of a dense and homogenous tissue on the luminal side of the grafts and a loser tissue formation in contact with the degraded scaffold on the outer surface (Fig. 2 A-B). The tissue was composed of cells and extracellular matrix as demonstrated by the presence of collagen in Masson's trichrome staining (Fig. 2 C). Immunohistochemistry staining showed the presence of α-smooth muscle actin positive cells in the inner layer of the vessel and CD31 positive cells forming a monolayer on the luminal side (Fig. 2 D-E). Moreover, the presence of a basement membrane underneath

the endothelial cells was demonstrated by the presence of collagen IV positive layer (Fig. 2 F).

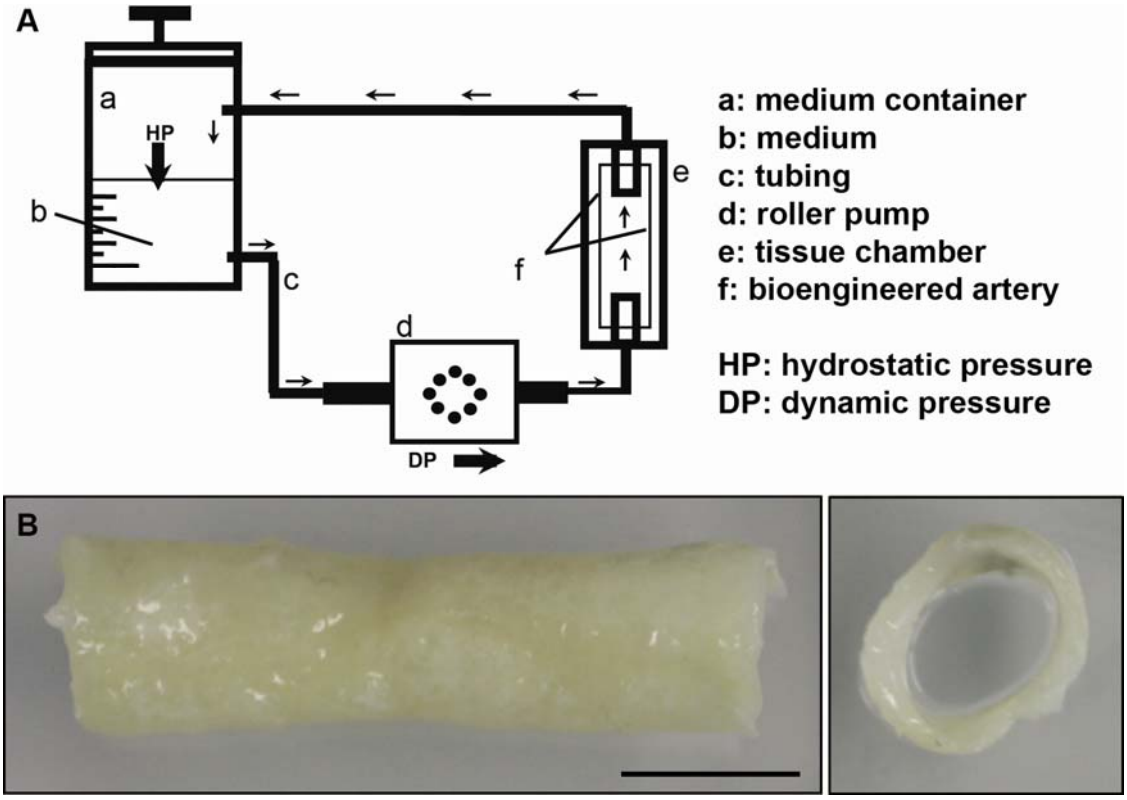

Figure 1. Bioreactor and macrostructure of the engineered artery equivalent. *A) Schematic view of the bioreactor set up. B) Macroscopic picture of the bioengineered artery demonstrated the presence of an open lumen after 5 weeks in culture as well as the formation of tissue on the luminal side of the graft. Bar represents 1 cm*.

The extracellular matrix composition of the graft was further examined with biochemical analyses of the presence of DNA, hydroxylproline (Hyp) or glycosaminoglycans (GAG) and the results were compared with the data obtained for a native human aorta. The GAG composition of the grafts was similar to the values observed in native tissues, 103 ± 23%. Interestingly the DNA and Hyp values represented only 39.5 ± 10% and 40 ± 5%, respectively of the native tissue values (see Fig. 3). In summary, these results demonstrated that the engineered artery equivalents mimicked the cellular structures as well as extracellular matrix composition of a native artery.

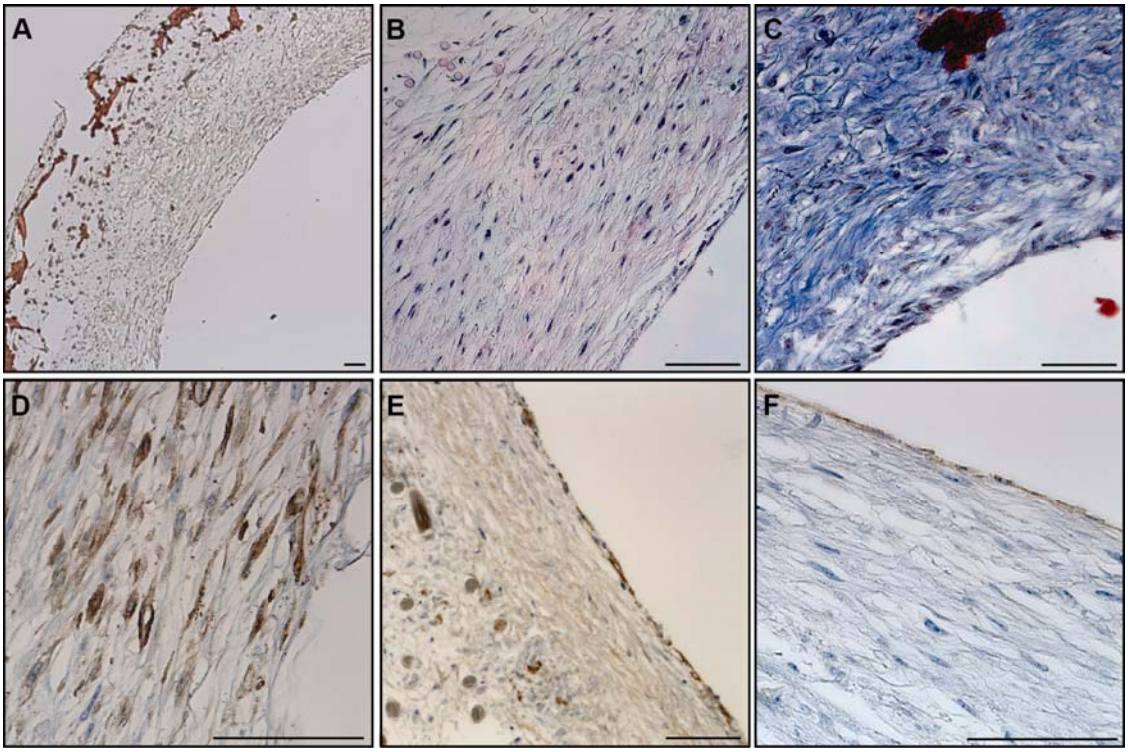

Figure 2. Histological structure of an engineered artery equivalent*. A) Haematoxylin-Sudan staining demonstrated the formation of tissue in and on the surface of a PGA/P4HB scaffold as well as the scaffold's partial degradation. B) H&E staining revealed dense tissue formation composed of cells and extracellular matrix. C) The secretion of collagen was observed after Masson's trichrome staining. The expression of* α*-SMA (D) confirmed the smooth muscle phenotype of the cells in the inner layer. Collagen IV positive staining (E) demonstrated the secretion of basement membrane and CD31 positive staining (F) confirmed the presence of an endothelial cell monolayer on the luminal side of the bioengineered artery equivalent. Bars represent 100* μ*m.*

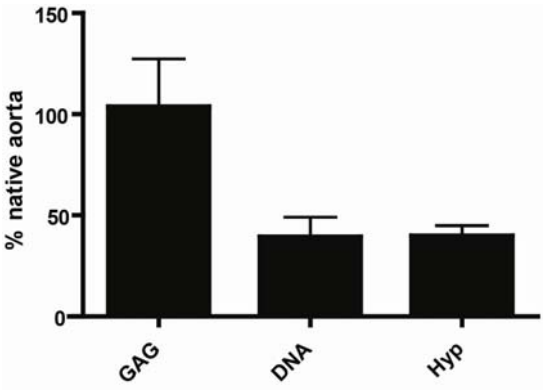

Figure 3. Quantification of extracellular matrix composition and cell number. *Total cell numbers, represented as DNA and total collagen secretion of collagen, represented as hydroxyproline (HYP) represented ≈ 50 % of the native human aorta. The glycosaminoglycan (GAG) content was similar to native aorta.*

Endothelial integrity assessment

Next, the integrity of the endothelium was analyzed using Evan's blue staining. As expected before endothelialisation, the Evan's blue dye was penetrating into the tissue and coloring evenly the bioengineered graft. (Fig. 4 A). One week after the seeding of endothelial cells, the sub-endothelial tissue was less intensively stained with Evan's Blue, indicating the beginning but not complete endothelialisation (data not shown). Two weeks after endothelial cell seeding, the tissue was not colored by Evan's blue any more indicating the presence of a tight endothelium (Fig. 4 B). To confirm the presence of tight junctions between the endothelial cells cryopreserved tissue was stained for tight junction protein 1 (ZO-1). The presence of ZO-1 expression on the luminal side of the graft was demonstrated two weeks after endothelial cell seeding (Fig. 4 C). Taken together these data suggest that the endothelium of the engineered artery equivalent formed a functional barrier isolating the sub-endothelial tissue from the luminal fluid as shown with the Evan's blue assay.

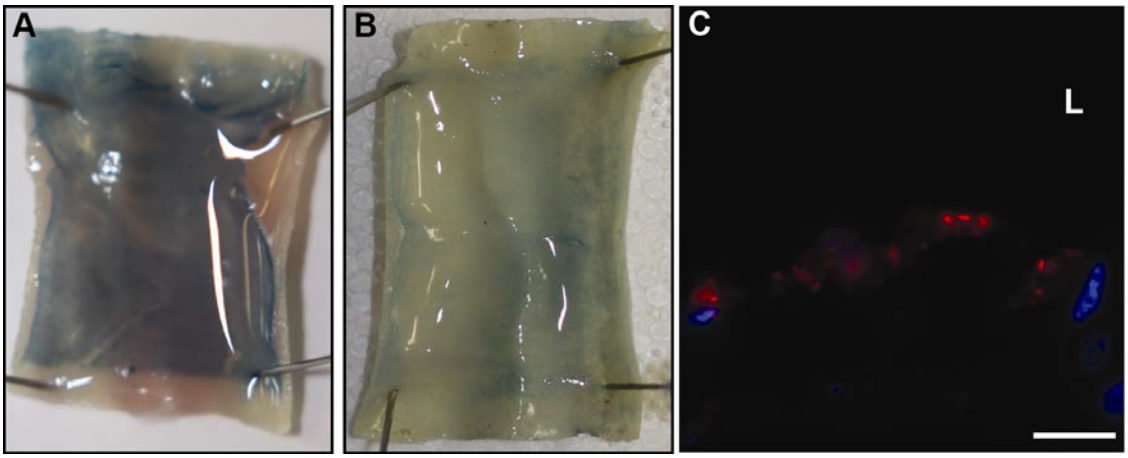

Figure 4. Endothelial cells integrity in the engineered artery*. The endothelial integrity was analyzed using Evan's blue staining (0.5% for 10 minutes). In the absence of endothelial cell (A) the tissue appeared evenly stained in blue. Two weeks after endothelialization (B) the endothelial barrier retained the absorption of Evan's blue and tissue appeared non-colored. The integrity of the endothelium was further analyzed by microscopy of cryosections for the expression of the tight junction protein (ZO)-1 (red) by endothelial cells (nuclei: blue). (C). Bar represents 50 μm and L: Lumen.*

Low-density lipoprotein insudation and accumulation in the tissue

The insudation and accumulation of LDL into the vasculature represents early events in atherosclerosis development. To investigate whether our engineered artery equivalent is suitable to mimic this step in atherogenesis, fluorescently labeled LDL was injected into the circulation loop of the dynamic graft culture. The tissue was collected after 2, 5 and 24 hours and analyzed by confocal imaging after fixation or cryopreserved for immunofluorescence. Confocal images were taken from the lumen of the graft and then – by changing the z-plane – by focusing deeper into the tissue. LDL was clearly localized intracellular in cytosolic vesicles within the luminal cell layer after 24 hours (Fig. 5 A). To verify the specific uptake of LDL, the engineered grafts were incubated with fluorescently labeled LDL in the presence of a 50 fold excess of non-labeled LDL, the signal was dramatically reduced (Fig. 5 B). This observation confirms the specificity of the LDL up-take into the engineered artery equivalent. Cryo-sections of the graft isolated at 2.5 and 24 hours demonstrated a time dependent uptake of LDL by the tissue. Indeed the signal emitted by the fluorescent LDL increased over time (Fig. 5 C & F). After 2.5 hours, LDL was mainly localized within the endothelial cell layer with minimal signal in the sub-endothelial space (white arrow Fig. 5 C-E). After 24 hours LDL was still localized in the endothelial cell layer but the sub-endothelial tissue revealed an increase of fluorescence (white arrow Fig. 5 F). Indeed in the sub-endothelial tissue LDL was found to accumulate either in spots (Fig. 5 G) or diffuse areas (Fig. 5 H). These results indicate the possibility to study trans-endothelial transport and sub-endothelial accumulation of LDL in our model.

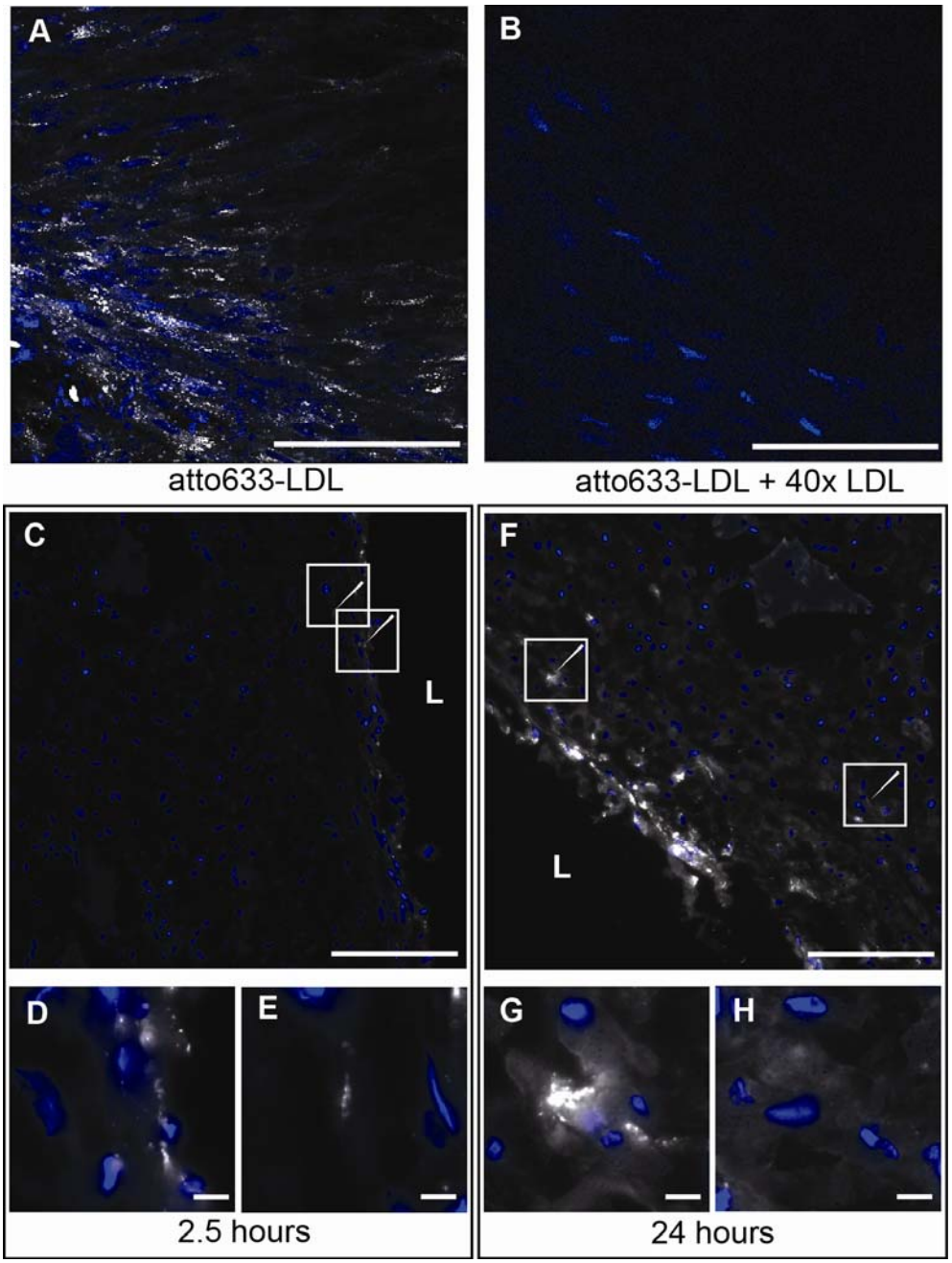

Figure 5. Insudation of LDL into the engineered artery. *The insudation of LDL into the tissue (blue: nuclei) was analyzed by confocal microscopy after incubation with 20 μg/ml of fluorescent LDL (white) for 24 hours and demonstrated the vesicular localization on the upper cell layer (A). The specificity of the signal was assessed by competition with 40-fold excess of non-labeled LDL (B). LDL localization in the tissue was further analyzed by microscopy of tissue cryosections and demonstrated the time-dependent uptake and the sub-endothelial localization of the LDL (C: 2.5 hours and F: 24 hours). Zoom in of interesting regions shown with the arrows are presented in D and E for 2.5 hours and F and G for 24 hours. Bars represent 200 μm (A-C, F) and 20 μm (D-E, G-H) and L: Lumen.*

Monocyte binding and insudation in engineering artery

To investigate if our model is suitable to analyses the attachment and trans-endothelium migration of monocytes into the interstitial vascular tissue, labeled monocytes were injected into the bioreactor circulation loop and tracked by confocal imaging and cryo-section analysis. The isolated monocytes were CD14 positive and after in vitro culture they express CD68 (data not shown). Monocytes were labeled with SNARF-1 dye after the isolation and the capacity of the labeled monocytes to bind to activated endothelial cells was verified in conventional cell culture (data not shown). The labeled monocytes were injected into the circulating medium of the graft in the absence or presence of 3 hours pre-treatment with TNFα. After 24 hours incubation, the attachment of the monocytes on the arterial graft was monitored by confocal imaging and demonstrated an increased number of monocytes adhering to the graft after TNFα pre-incubation (white arrows; Fig. 6 A-B). Moreover after 24 hours pre-incubation of the graft with LDL, the monocytes attached to the endothelium to a similar extent as compared with the TNFα pre-stimulation (Fig. 6 C). Interestingly, if the monocytes were added simultaneously with the LDL no increase of monocyte adhesion could be observed compared to the non-stimulated grafts (data not shown). In addition, the attachment of the monocytes to the scaffold alone was investigated and demonstrated no adhesion. To quantify monocyte adhesion, monocytes having remained in the circulation after 24 hours were counted and compared to the monocyte number of the control group (monocyte perfusion of scaffold only). The numbers of monocytes in the circulation after TNFα and LDL pre-stimulation 17.5 $\pm$ 3.0 % and 20.8 $\pm$ 3.5 % respectively were not statically different ($p = 0.226$). However, they were significantly lower compared to the not stimulated engineered arteries (33,7 $\pm$ 2,8 %), $p < 0.001$ for TNFα and $p < 0.001$ for LDL. These results indicate that significantly more monocytes were bound under stimulated conditions. Microscopy of the cryo-sectioned tissue after LDL pre-incubation demonstrated the presence of monocytes adhering to the endothelial surface and migrating into the tissue (Fig. 6 E & G). In addition, monocytes were also identified in the tissue after LDL pre-treatment (Fig. 6 F & H-I). These results indicate that our engineered artery equivalent represents a suitable tool to study the attachment and

transmigration of monocytes into the artery, which represents a key process in the development of atherosclerosis in vivo.

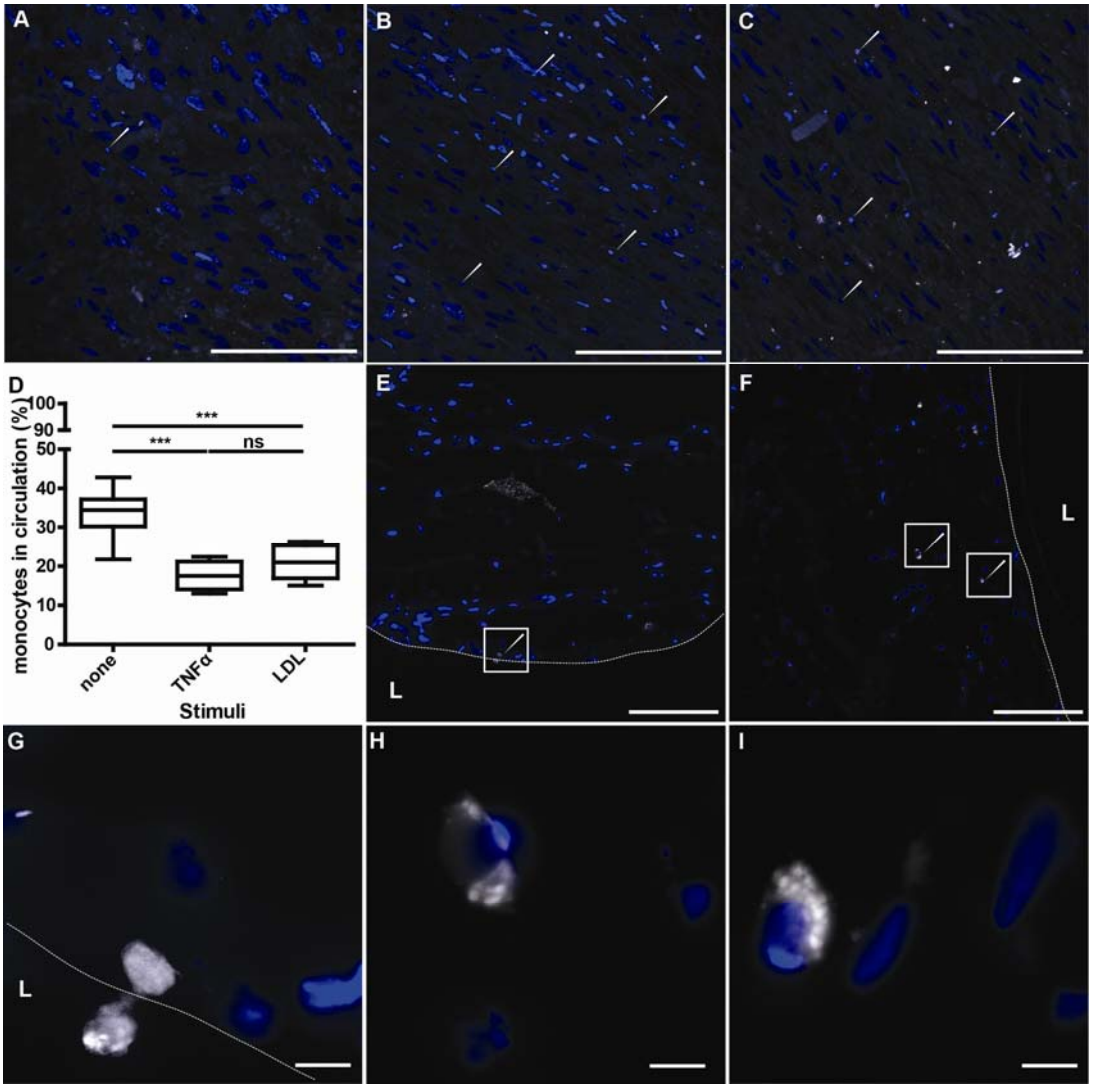

Figure 6. Endothelial monocyte adhesion and transmigration migration in the engineered artery. *After pre-treatment in the absence (A, D) or the presence of 3 hours TNFα (10 ng/ml) (B, D) or 24 hours LDL (20 μg/ml) (C-I) 1x10^6 fluorescently labeled monocytes (white) per ml were injected into the circulation loop and circulated for 24 hours. Tissues were analyzed by confocal microscopy and after cryosectionning. In addition, monocytes remaining in the circulation were counted. More monocytes (white arrows) adhered after pre-treatment with TNFα (B) or LDL (C) compared to the not stimulated (A). Less monocytes remained into the circulation after TNFα or LDL pre-treatment compared to the absence of stimuli (D). Monocytes adhesion and migration in the tissue was further analyzed by microscopy of cryosections after LDL pre-treatment. Microscopic observations demonstrated adhesion and migration of monocytes through the endothelium (dash line) (E, G) and accumulation of monocytes into the tissue (F, H-I). Bars represent 200 μm (A-C, E-F) and 20 μm (G-I).*

High density lipoprotein insudation in engineered artery.

HDL is an important key player in the development of atherosclerosis development. Indeed HDL is believed to have a major anti-atherosclerotic potential by removing the excess cholesterol out of the macrophages in the arterial wall. In this context, the trans-endothelial transport of HDL remains a potentially limiting step to be elucidated [22]. The ATP binding cassette (ABC) G1, the scavenger receptor (SR)-BI and endothelial lipase (EL) were demonstrated to participate in the trans-endothelial transport of HDL in cell culture [23]. In order to analyses HDL transport across the endothelium the expression of the known HDL binding proteins were investigated by immunofluorescence stainings. Specific stainings with antibodies against ABCG1, SR-BI and EL demonstrated the high expression of those markers by both HUVECs and HUMFBs in the engineered artery equivalent in a comparable pattern as in a native artery (Fig. 7). To further investigate whether our model is suitable to analyses the transport of HDL through endothelial cells, we incubated the bioengineered graft with fluorescently labeled HDL. Similar as described for LDL, cryosection of the harvested engineered arteries demonstrated the presence of HDL in the endothelial cells as well as the accumulation of HDL in the sub-endothelial space (Fig. 8A). In addition, the distribution of HDL was analyzed by confocal microscopy. Interestingly, HDLs were localized either in intracellular vesicles or as extracellular diffused material if incubated with engineered arteries under flow conditions (Fig. 8B). However, under static non-flow conditions labeled HDL was recovered in intracellular vesicles only (Fig. 8 C & D). These differences between static (non-flow) cell culture conditions and the flow conditions of our engineered arteries demonstrates the potential of the proposed model to study HDL transport under in vitro in conditions which are closer to the native situation.

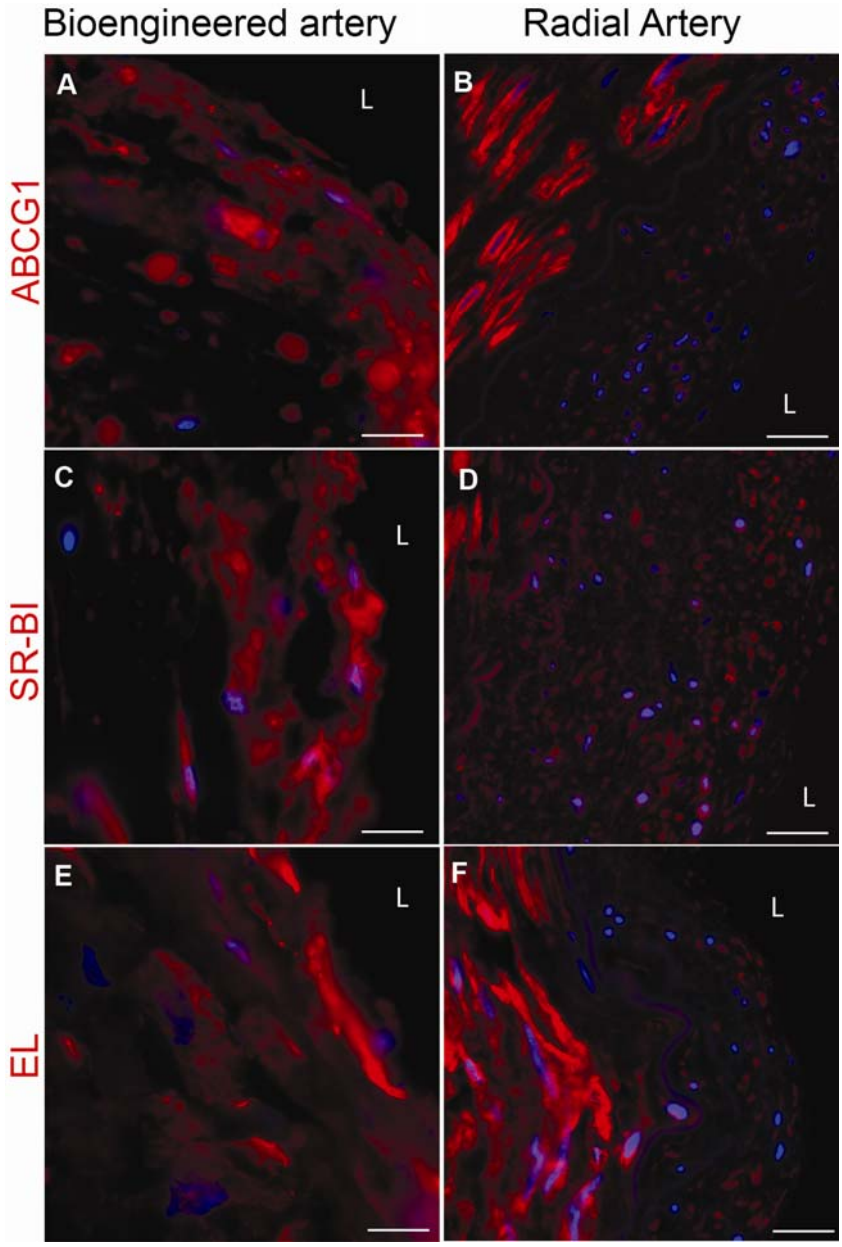

Figure 7. Expression of ABCG1, SR-BI and EL in the bioengineered artery in comparison to native artery*. The expression of ABCG1 (red) (A), SR-BI (red) (C) and EL (red) (E) was assessed by immunofluorescence staining after cryosection of the engineered artery and compared to a native radial artery (B, D, F) (blue: nuclei). Bars represent 50 μm.*

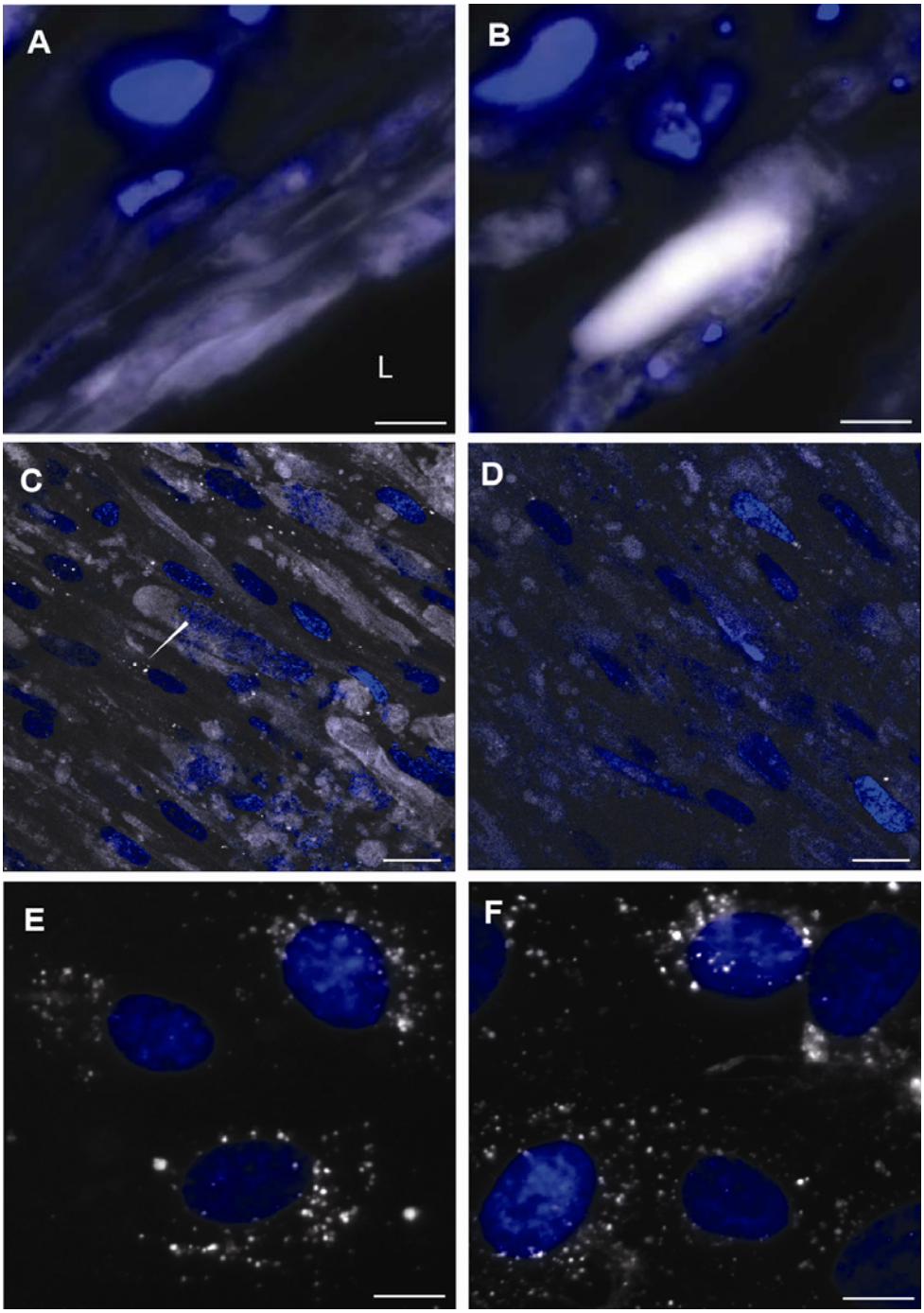

Figure 8. HDL insudation in the engineered artery equivalent*. 25 μg/ml of HDL was circulated for 24 hours. The insudation of HDL (white) was assessed after cryosection of the tissue on the luminal side (A) and in the tissue (B) (blue: nuclei). HDL localization in the tissue was further analyzed by confocal microscopy at 2.5 um (C) and 16 um (D) deep in the tissue. The arrow shows the intracellular vesicular localization. The up-take of HDL (white) into HUVECs (E) and UCMFB (F) was analyzed in regular cell culture after 24 hours incubation with 25 μg/ml. Bars represent 50 μm and L: Lumen*

Discussion

Given its substantial negative socioeconomic impact, atherosclerosis has been intensively studied in the last decades. In particular, many efforts have been undertaken to develop an adequate ex vivo model for the biofunctional large-scale assessment of ethiopathogenetic phenomena in vitro. The development of endothelial and smooth muscle cell co-culture systems for the assessment of monocyte transmigration and accumulation in the artery intima, representing a key step in the pathogenesis of atherosclerosis, was already proposed in the early 1990's [5,6]. However, as already emphasized by Dorweiler et al., these co-culture systems allow neither for long term in vitro culture nor histology and extracellular matrix analyses [8]. Their own co-culture system on a fibrin gel allowed longer culture times for up to 6 weeks and hence represented a major step forwards the in vitro modeling of atherosclerosis[8,9]. Nevertheless, in spite of this improvement, also this approach was missing a major physiologic aspect evident in the biology of native arteries - that is the presence of (pulsatile) blood flow in a lumen. This seems most important because atherosclerotic lesions prevail at the site of altered blood flow as first reported by Caro et al. [24].

Therefore, the present study addressed the question whether bioengineered artery equivalents - as previously developed as therapeutic replacements for patients with structural cardiovascular disease [10–13]- are suitable for modeling essential pathogenic phenomena of atherosclerosis in vitro. In particular, the pulsatile native-like flow environment could represent a major advantage in this regard when compared to previous studies using static cell culture environments.

As previously shown, histological analyses of the bioengineered grafts demonstrated a structure similar to the layered microstructure of the native artery [10,14,25]. These constructs were equally covered with a multi-layer of α-smooth muscle actin positive cells indicating the development of a media-like layer. The presence of secreted extracellular matrix components, such as collagen and glycosaminglycans demonstrated the in situ functionality of smooth muscle cells in the tissue. However, the biochemical DNA and the collagen quantification, revealed lower values than found in the native

artery. This phenomenon has been previously described and supposed to be due to the relatively short in vitro culture periods [10]. This is further supported by the experiments in large animal models, where tissue engineered arteries were exposed to longer in vitro and in vivo conditioning and eventually showed higher ECM values [14]. The positive layer of collagen IV suggested the presence of a basement membrane, that is secreted by the endothelial cells and separates them from the sub endothelial space [26]. In addition, the luminal side was covered with a monolayer of endothelial cells on top of the basement membrane. Another major function of the endothelium is the formation of a tight barrier between the blood and the interstitial vascular tissue. In our engineered grafts, the integrity of the endothelial layer was confirmed by its impermeability for Evan's blue stain as well as by the expression of the ZO-1.

The microscopic demonstration of labeled LDL in intracellular vesicles and the sub endothelial space indicate the uptake and resecretion of LDL. According to the response-to retention model, the accumulation of LDL in the sub-endothelial space is a crucial step in the formation of atherosclerotic lesions [27]. Besides the accumulation of LDL, inflammatory processes play an indispensable role in the pathogenesis of atherosclerosis, for example by endothelial cell activation and subsequent monocyte adhesion [28]. Therefore, we applied TNFα to analyses monocyte adhesion to the endothelium. The observed significant increase of monocyte adhesion after stimulation with either TNFα or LDL hence provides another indication that our model is feasible to study pathogenic events.

The trans-endothelial transport of HDL in the arterial wall for removal of cholesterol accumulation is a key step in the reverse cholesterol transport [3]. In addition, apoA-I - the major apolipoprotein of HDL - was found to be present in atherosclerotic plaque by recent investigations [29]. In agreement with these histological descriptions and our previous findings of trans-endothelial HDL transport in transwell cell culture we found immunofluosecently labeled HDL both in endothelial cells and the sub-endothelial space of our 3D model.

In conclusion, we demonstrate for the first time the feasibility to engineer a dynamic 3D human artery equivalent for the investigation of fundamental processes involved in the development of atherosclerosis in vitro. In the contrary to previous investigations, this model combines the engineering of a native-like multilayer 3D architecture of the vascular wall with a lumen and the applicability of native-analogous pulsatile flow profiles. Thereby, this unique model mimics the native-like vascular environment and may therefore also allow for more representative in vitro investigations with higher predictive value for human pathologies in vivo. In addition, this model may also allow for large scale in vitro drug screening experiments – in particular by enabling the specific modulation of several parameters (i.e. flow, pressure, LDL, HDL, type of immune cells, etc). Thus, this model may ultimately help to significantly reduce the necessity of animal experiments in the field of atherosclerosis research. We therefore believe that our model represents an important step towards the development of a native-like large-scale atherosclerosis in vitro disease model, which may help to improve our understanding of this important pathology.

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Disclosures

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Chapter

5

General discussion and conclusions

Atherosclerosis remains the major cause of morbidity and mortality in westernize-countries, with an approximated cost of € 192 billion per year [1]. A better understanding of the development of the disease and the mechanisms of cholesterol clearance (RCT) may help to developed new treatments and to reduce these costs. In the present dissertation the role of inflammatory cytokines on the transport of HDL and lipid-free apoA-I through endothelial cells, the first step in RCT, is studied together with the development of a novel three dimensional engineering artery model for atherosclerosis research.

During RCT, HDL and lipid-poor or lipid-free apoA-I remove cholesterol out of the macrophage foam cells and carry it back to the liver for disposal. Several lines of evidence suggest that the macrophage-specific cholesterol efflux is not taking place in the blood circulation but rather in the arterial wall compartment [2]. Therefore HDL and apoA-I have to cross the endothelium to get access to the cholesterol. Previously we demonstrated in aortic endothelial cells cultivated in an in vitro transwell system that HDL and apoA-I are transcytosed through endothelial cells in a process involving ABCG1 and SR-BI for HDL and ABCA1 and ectopic β-ATPase for apoA-I [3–6]. In the present studies, the trans-locations of HDL an apoA-I were studied in condition mimicking inflammation that is a hall-mark of atherosclerosis. To this end the cells were stimulated with IL-6, TNFα and IL-1β, the major cytokines found within the plaque [7,8].

Interestingly IL-6 induced the transport of HDL through endothelial cells whereas TNFα and IL-1β reduced the transport of both HDL and apoA-I. These cytokines were previously reported to have adverse effects on atherogenesis. Whereas IL-1β and TNFα are pro-atherogenic, the role of IL-6 remains so far un-clear [9–11]. Indeed both mice overexpressing IL-6 and IL-6$^{-/-}$ mice develop larger atherosclerotic lesions [12,13]. Moreover Frisdal et al. revealed that IL-6 induces macrophage cholesterol efflux [14]. These results suggest a possible positive effect of IL-6 in RCT from macrophages correlating with our results. In addition to the anti-inflammatory properties of IL-6, the signaling pathways of IL-6, IL-1β and TNFα are different [15–17]. Analyzing the pathways after these cytokine stimulations will permit to better understand the differences

observed in the transport of HDL through endothelial cells in between IL-6, IL-1β and TNFα.

The trans-endothelial transport of HDL after cytokine stimulations permitted to identify endothelial lipase (EL) as a novel HDL binding protein in endothelial cells, facilitating trans-endothelial HDL transport (Fig. 1).

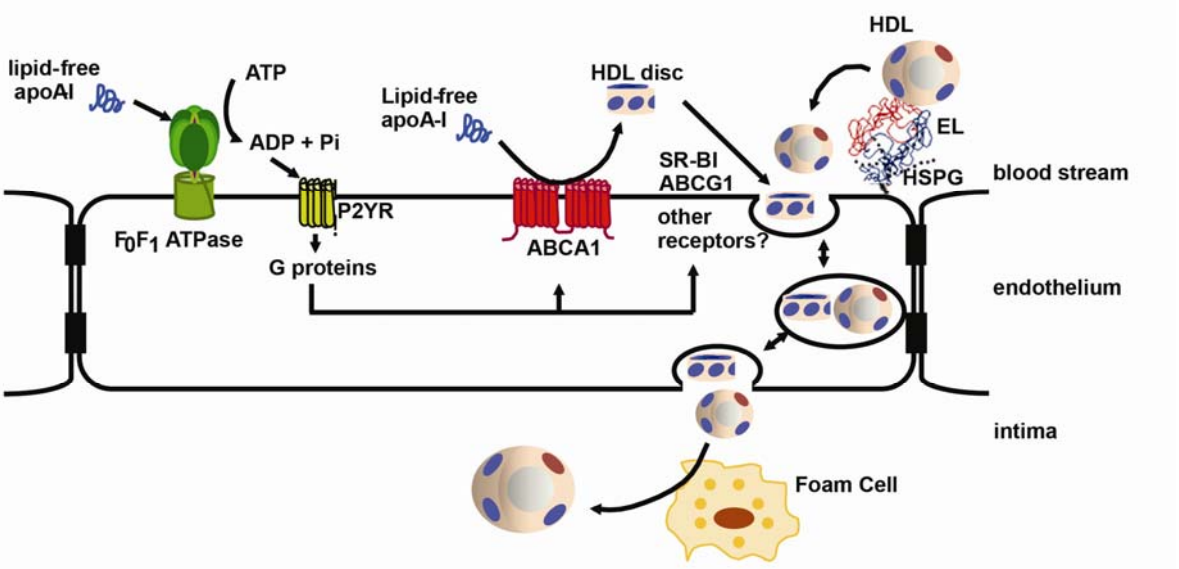

Figure 1. Trans-endothelial transport of HDL and apoA-I. *EL present on heparan sulfate proteoglycans (HSPG) binds and partially hydrolyses HDL on the cell surface. HDL is then further internalized dependently of SR-BI and ABCG1. Lipids-free apoA-I binds to ABCA1 and gets lipidated and further internalizes by ABCA1 independent mechanisms. In addition lipid-free apoA-I binds to the ectopic β-ATPase and signals via P2Y receptor to induce internalization of lipidated apoA-I and HDL.*

Interestingly EL was previously reported to induce HDL binding to hepatocytes independently of its catalytic activity via bridging of HDL [18]. In the present study we reported that the catalytic activity is essential for HDL transport however it is not clear if EL has bridging function in endothelial cells. To answer this question endothelial cells transfected with a non-catalytic active EL will permit us to better understand the bridging role of EL in endothelial cells. In addition Nijstad et al. demonstrated that hepatic selective uptake by SR-BI involves a size reduction of HDL by EL and proposed that the two proteins are working sequentially in hepatocytes [23]. The cooperative effect of EL with either SR-BI or ABCG1 in endothelial cells s is not yet clear. Co-silencing or

pharmacological inhibition of EL and SR-BI or ABCG1 will offer us the possibility to further analyze the possible sequential action of EL and SR-BI or ABCG1.

The transports of HDL and apoA-I were performed in aortic endothelial cells cultivated in transwell system and therefore the physiological relevance of the model can be questioned. Therefore an ex-vivo artery equivalent was engineered with human endothelial cells and myofibroblasts. In this aortic equivalent physiological luminal flow was introduced which was missing in previous studies [24–27]. Blood flow and in particular disturbed blood flow causing shear stress is known to be important to induce atherosclerotic plaque formation [28].

In the present thesis we reported the feasibility to monitor the early steps in atherogenesis, meaning the insudation and sub-endothelial retention of LDL as well as the binding of monocytes to activate endothelial cells and their further transmigration to the tissue under flow conditions. Due to the production complexity and to the limited artery equivalents produced, the present study represents a proof of principle and further experiments with time and dose kinetics will be necessary to validate the present atherosclerotic model.

Although the flow in our model is not equivalent to native conditions, the model represents a major step forward in modeling atherosclerosis ex-vivo. Future experiments with flow and pressure kinetics will be of particular interest in further validation experiments. In addition analysis of shear stress by branching in the engineered graft or formation of a tissue bulk to partially block the flow will provide further information. Moreover, the atherosclerosis model will make possible to study other atherogenic components e.g. lymphocyte interactions, the effect of glycation end products, reactive oxygen species on the endothelium, the endothelial repair process in normal and inflammatory conditions. Last but not least it open the possibility to investigate in more detail the trans-endothelium transport of HDL or apoA-I under normal or pathological conditions so that model will provide answers relevant for RCT.

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